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(54) Title: <i>RIN2, A NOVEL INHIBITOR OF RAS-MEDIATED SIGNALING</i>															
<table border="1"> <thead> <tr> <th>Examples of normal responses regulated by Ras-dependent signaling</th> <th>Settings in which it is desirable to decrease such responses</th> <th>Settings in which it is desirable to increase such responses</th> </tr> </thead> <tbody> <tr> <td>Mast cell mediator production</td> <td>Asthma, hay fever, atopic dermatitis</td> <td>Immunity to pathogens</td> </tr> <tr> <td>T cell function</td> <td>Graft rejection, T cell dependent diseases (certain autoimmune diseases, contact dermatitis, graft-versus-host disease)</td> <td>HIV infection, certain other infections, immunosuppressions associated with cancer therapies</td> </tr> <tr> <td>Cell proliferation</td> <td>Cancer, proliferation of vascular wall cells in arteriosclerosis</td> <td>Wound healing, angiogenesis (e.g. to improve ischemia, re-epithelialization), nerve regeneration</td> </tr> </tbody> </table>				Examples of normal responses regulated by Ras-dependent signaling	Settings in which it is desirable to decrease such responses	Settings in which it is desirable to increase such responses	Mast cell mediator production	Asthma, hay fever, atopic dermatitis	Immunity to pathogens	T cell function	Graft rejection, T cell dependent diseases (certain autoimmune diseases, contact dermatitis, graft-versus-host disease)	HIV infection, certain other infections, immunosuppressions associated with cancer therapies	Cell proliferation	Cancer, proliferation of vascular wall cells in arteriosclerosis	Wound healing, angiogenesis (e.g. to improve ischemia, re-epithelialization), nerve regeneration
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(57) Abstract															
<p>Novel gene, <i>Rin2</i>, and encoded protein are disclosed which can inhibit the functional response induced by Ras-dependent signaling pathways are disclosed. Methods of inhibiting or enhancing Ras-dependent signaling and methods of treatment utilizing <i>Rin2</i> are also disclosed.</p>															

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-1-

RIN2, A NOVEL INHIBITOR OF RAS-MEDIATED SIGNALING

RELATED APPLICATIONS

5       The present application is a Continuation-in-Part  
of U.S. Application Serial No. 08/942,819, filed October  
2, 1997. The present application also claims the  
benefit of U.S. Provisional Application No. 60/058,520,  
filed September 11, 1997. The teachings of the prior  
10 applications are incorporated herein in their entirety.

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15 Health. The United States government has certain rights  
in the invention.

BACKGROUND OF THE INVENTION

The Ras family of small guanine-nucleotide binding  
20 proteins plays a pivotal role in many intracellular  
signal transduction pathways, including those which  
regulate cellular growth and differentiation and those  
which contribute to cell activation (Bourne et al.,  
*Nature* 348:125-132 (1990); Marshall, *FASEB J.*  
25 9:1311-1318 (1995)). Moreover, many different receptors  
expressed on the surface of diverse cell types can  
result in the activation of signal transduction pathways  
that are importantly influenced by Ras, and these  
pathways in turn determine whether, and to what extent,

-2-

these cells respond to such cell surface receptor-dependent activation by proliferating, differentiating (i.e., developing new functional characteristics), and/or expressing specific functions (Figure 1).

5        Depending on the circumstances, these "down-stream" consequences of the activation of Ras-dependent signal transduction pathways can have either adaptive (physiological) or maladaptive (pathological) consequences. For example, controlled Ras-dependent  
10      cellular proliferation is required for wound healing, whereas poorly regulated Ras-dependent cellular proliferation can result in the development of cancer and other neoplasms. Similarly, appropriate Ras-dependent cell secretion of histamine, serotonin,  
15      cytokines and other mediators can be important for host defense against parasites and other pathogens, whereas the inappropriate activation of these same pathways, for example, by a reaction to a bee-sting in patients who are allergic to components of bee venom, can lead to  
20      fatal anaphylaxis. Thus, Ras represents a major regulator of many of the most fundamental biological processes involved in both health and disease.

         The mechanism by which Ras regulates such processes, through interactions with other intracellular  
25      molecules, is quite complex. Ras proteins are membrane-associated proteins that cycle between an active GTP-bound form and an inactive GDP-bound form. As illustrated in Figure 1, evidence is accumulating for the existence of many different classes of positive or  
30      negative regulators of Ras and positive or negative Ras signaling effectors, all of which, by definition, are thought to interact directly with the active GTP-bound

-3-

form of Ras to influence cellular signaling for growth, differentiation and expression of function (Boguski and McCormick, *Nature* 366:643-654 (1993); Marshall, *FASEB J.* 9:1311-1318 (1995); Marshall, *Curr. Opin. Cell Biol* 8:197-204 (1996)).

For example, some of the best-characterized Ras regulators include the GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (Boguski and McCormick, *Nature* 366:643-654 (1993)). The GAPs represent a family of Ras-binding proteins which stimulate the intrinsic rate of Ras GTP hydrolysis and thus negatively regulate the Ras-induced signaling by accelerating the conversion of active GTP-bound form of Ras to the inactive GDP-bound form. Recent studies have identified several GAPs specific for Ras proteins, which include p120-Ras GAP, neurofibromin (the protein encoded by the neurofibromatosis type 1 (NF1) gene), Gap1, Ral-GDS, Rsbs 1, 2, and 4, Rin1, MEKK-1, and phosphatidylinositol-3-OH kinase (PI3K) (Boguski and McCormick, *Nature* 366:643-654 (1993)).

In contrast to Ras regulators, which function primarily by influencing the amount of Ras which is in the GTP-bound active, as opposed to the GDP-bound inactive, form, Ras effectors are thought to influence the ability of active, GTP-bound Ras to initiate signaling (Figure 1). In the case of many Ras-interacting proteins which can influence the intensity of Ras-dependent signaling, it is not yet clear to what extent they function as effectors as opposed to regulators; such proteins can therefore be called Ras regulators/effectors (Boguski and McCormick, *Nature* 366:643-54 (1993); Han and Colicelli, *Mol. Cell. Biol.*

-4-

15:1318-1323 (1995); Marshall, *FASEB J.* 9:1311-1318 (1995); Marshall, *Curr. Opin. Cell. Biol.* 8:197-204 (1996)).

Ras is important in critical cell signaling events  
5 in many cell types. For example, mast cells are important effector cells in IgE-dependent immune responses and allergic diseases (Galli, *New Engl. J. Med.* 328:257-265 (1993)), and mast cells also contribute to host defense against parasites and bacteria  
10 (Echtenacher et al., *Nature* 381:75-77 (1996); Malaviya et al., *Nature* 381:77-80 (1996); Galli and Wershil, *Nature* 381:21-22 (1996)). Mast cells reside in virtually all vascularized tissues and express on their surface the high affinity receptor for IgE (Fc $\epsilon$ RI).  
15 Aggregation of Fc $\epsilon$ RI in mast cells by the interaction of receptor-bound IgE with specific multivalent antigen triggers the functional activation of mast cells, which results in the release of a spectrum of biologically active mediators (Ravetch and Kinet, *Ann. Rev. Immunol.* 9:457-492 (1991); Galli, *New Engl. J. Med.* 328:257-265 (1993); Beaven and Metzger, *Immunol. Today* 14:222-226 (1993)). Thus, mast cells activated by Fc $\epsilon$ RI-dependent mechanisms undergo degranulation, resulting in the release of preformed mediators, such as serotonin (5-HT)  
20 and/or histamine, the metabolism of arachidonic acid, leading to the release of newly synthesized lipid mediators, and the transcription, translation and secretion of several cytokines (Gordon et al., *Immunol. Today* 11:458-464 (1990); Galli, *New Engl. J. Med.* 328:257-265 (1993); Paul et al., *Adv. Immunol.* 53:1-29 (1993)).

-5-

Knowledge of the signaling pathways which result in the Fc $\epsilon$ RI-dependent secretion of mast cell mediators is increasing. In mast cells and basophils, a type of circulating leukocyte which shares many biochemical and functional characteristics with mast cells (Galli, *New Engl. J. Med.* 328:257-265 (1993)), the Fc $\epsilon$ RI receptor is a tetrameric complex comprised of a single 45 kDa  $\alpha$  chain, which binds the Fc portion of IgE, a single 30 kDa  $\beta$  chain, and a homodimer of two 10 kDa  $\gamma$  chains (Ravetch and Kinet, *Ann. Rev. Immunol.* 9:457-492 (1991); Beaven and Metzger, *Immunol. Today* 14:222-226 (1993)). The  $\beta$  and  $\gamma$  chains contain immunoreceptor tyrosine-based activation motifs (ITAM) which couple the receptor to the src family of protein tyrosine kinases (PTK) p56lyn and p72syk (Beaven and Baumgartner, *Curr. Opin. Immunol.* 8:766-772 (1996)). The Fc $\epsilon$ RI-dependent activation of these PTKs in turn activates various downstream effector pathways, including those involving PLC $\gamma$ 1 and the MAP kinase pathway (Beaven and Baumgartner, *Curr. Opin. Immunol.* 8:766-772 (1996)).

Recent studies have shown that the crosslinking of Fc $\epsilon$ RI in mast cells by IgE and specific antigen also results in the activation of Ras and of the associated Shc-Grb2-Sos pathway, which precedes Ras activation, and that the activation of this pathway is dependent on Syk (Jabril-Cuenod et al., *J. Biol. Chem.* 271:16268-16272 (1996)). These results suggest that in Fc $\epsilon$ RI-activated mast cells, as in T cells and B cells (the major types of lymphocytes responsible for cellular and humoral immunity) which have been activated via the T cell receptor or B cell receptor, respectively, the Shc-Grb2-

-6-

Sos pathway can activate the MAP kinase pathway via the activation of Ras.

An effector pathway of Ras mediated by the Raf-1/Erk-activating kinases (MEKs)/Erk-MAP kinases 5 cascade has been well-characterized in numerous systems (Treisman, *Curr. Opin. Cell Biol.* 8:205-215 (1996)), and this Ras-mediated pathway is important in the mast cell activation and mediator secretion that is induced by IgE- and antigen-dependent aggregation of Fc $\epsilon$ RI in these 10 cells (Tsai et al., *Eur. J. Immunol.* 23:3286-3291 (1993); Offermanns et al., *J. Immunol.* 152:250-261 (1994); Hirasawa et al., *J. Immunol.* 154:5391-5402 (1995)). In addition, recent studies with rat RBL2H3 mast cells have shown that the Fc $\epsilon$ RI induction of Ras 15 activation leads to transcriptional activation mediated by the transcription factors Elk-1 and the nuclear factor of activated T cells (NFAT) (Turner and Cantrell, *J. Exp. Med.* 185:43-53 (1997)). These Ras-dependent signaling pathways in mast cells appear to be complex. 20 Thus, activation of the Raf-1/MEK/Erk cascade appears to be necessary and sufficient for the activation of Elk-1 activity in mast cells. However, the induction of NFAT by Fc $\epsilon$ RI in mast cells is also mediated in part by Rac-1, which is a putative Ras effector and a member of 25 the Rho family of GTP binding proteins (Turner and Cantrell, *J. Exp. Med.* 185:43-53 (1997)). Thus, the specific signaling pathways involved in Fc $\epsilon$ RI-mediated mast cell activation are still unclear.

30 SUMMARY OF THE INVENTION

As described herein, mRNA differential display was used to identify the cDNA for a novel gene, *rin2* (also

- 7 -

called Rabex-5). As also described herein, expression of *rin2* was shown to be rapidly increased in mouse mast cells activated through Fc $\epsilon$ RI. Transfection of mouse mast cells with an antisense *rin2* expression vector, 5 which resulted in antisense inhibition of Rin2 expression, potentiated the Ras-mediated intracellular signaling responses that were induced by Fc $\epsilon$ RI aggregation, such as the induction of *c-fos* expression and the activation of Erk-MAP kinase, JNK and p38 MAP 10 kinase activity. In addition, antisense inhibition of *rin2* expression in mouse mast cells significantly enhanced the amounts of preformed mediator (serotonin) and cytokine (IL-6) released from these cells upon Fc $\epsilon$ RI-dependent stimulation, suggesting that Rin2 exerts 15 its effects by down-regulating the functional responses elicited by Fc $\epsilon$ RI aggregation in mast cells. These results support the role of Rin2 as a novel negative regulator/effectector of Ras in mast cells and indicate that Rin2 interacts directly the mammalian H-Ras 20 protein.

Moreover, as also described herein, expression of *rin2* is also increased in mast cells stimulated via activation of their major growth factor receptor (i.e., c-kit), in PC12 adrenal pheochromocytoma cells activated 25 via the receptor for nerve growth factor (NGF) (i.e., TrkA), and in T cells activated via the T cell receptor (TCR). These findings support the role of Rin2 as a novel general negative regulator/effectector of Ras and Ras-dependent signaling pathways in diverse cell types 30 which have been activated via distinct cell surface receptors (Figure 2). In addition, the enhancement or inhibition of Rin2 expression or function, in certain

settings, resulting in the reduction or enhancement, respectively, of Ras-dependent signaling in these settings, may have benefit in diverse clinical problems, some examples of which are shown in Figure 2.

5 As also described herein, the SY-A (Rin2) clone has been used as a probe to screen a HT-29 human adenocarcinoma cDNA library. Two overlapping cDNA clones (HRIN2-1B1 (1.3 kb) and HRIN2-2B1 (2.9 kb)) have been identified which are highly homologous to that of  
10 the mouse Rin2 clone and which together represent the full-length human Rin2 (Rabex-5) cDNA sequence. The orientation of these overlapping clones is shown in Figure 9. The nucleotide sequence of each clone is shown in Figures 10A-B and 11A-C, respectively.

15 Accordingly, this invention pertains to isolated Rin2 protein, which down-regulates the functional responses elicited by Ras-dependent signaling pathways. In one embodiment, Rin2 down-regulates the functional responses elicited by Fc $\epsilon$ RI aggregation in mast cells  
20 (e.g., mammalian mast cells), including down-regulating the amounts of preformed mediator (serotonin) and cytokine (IL-6) released from these cells upon Fc $\epsilon$ RI-dependent stimulation. In another embodiment, Rin2 down-regulates functional responses in mast cells  
25 stimulated via activation of their major growth factor receptor (i.e., c-kit). In another embodiment, Rin2 inhibits cell proliferation in PC12 adrenal pheochromocytoma cells stimulated through the nerve growth factor receptor (i.e., TrkA). In a further  
30 embodiment, Rin2 inhibits functional responses in T cells activated through the T cell receptor.

-9-

In one embodiment, isolated Rin2 protein has the amino acid sequence of SEQ ID NO: 2. In one embodiment, the Rin2 protein is a portion of SEQ ID NO: 2 which is sufficient for Rin2 activity, e.g., binding or enzymatic 5 activity. Also included within the meaning of Rin2 protein or polypeptide are polypeptides which are less than the amino acid sequence of SEQ ID NO: 2 and have Rin2 activity, as well as proteins whose amino acid sequence is substantially similar to that of SEQ ID NO: 10 2 and have Rin2 activity.

In another embodiment, Rin2 protein is a derivative possessing substantial sequence identity with the endogenous Rin2 protein. In particular embodiments, the Rin2 protein is purified to homogeneity or is 15 substantially free of other proteins.

The invention also pertains to an isolated nucleic acid molecule or nucleotide sequence (*rin2*) which encodes Rin2 protein or polypeptide, or a portion of SEQ ID NO: 2 which is sufficient for Rin2 activity. In one 20 embodiment, the encoded Rin2 protein is a derivative possessing substantial sequence identity with the endogenous Rin2 protein. In a particular embodiment, the isolated nucleic acid molecule encodes Rin2 protein with the same amino acid sequence as endogenous Rin2 25 protein. In another embodiment, the isolated nucleic acid molecule has the same nucleotide sequence as the endogenous gene encoding Rin2 protein. In one embodiment, the isolated nucleic acid molecule has the nucleotide sequence of SEQ ID NO: 1. In another 30 embodiment, the isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 12 and/or the nucleotide sequence of SEQ ID NO: 13. In a further

-10-

embodiment, the isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 14 and/or the nucleotide sequence of SEQ ID NO: 15. In one embodiment the isolated *rin2* nucleic acid molecule comprises the 5 nucleotide sequence of one or more of SEQ ID NOS: 12, 13, 14 and 15.

The invention also relates to DNA constructs comprising the nucleic acid molecules described herein, operatively linked to a regulatory sequence, and to 10 recombinant host cells, such as bacterial cells, fungal cells, plant cells, insect cells and mammalian cells, comprising the nucleic acid molecules described herein operatively linked to a regulatory sequence. The invention also relates to a method for preparing a Rin2 15 polypeptide, comprising culturing a recombinant host cell described herein.

The invention also pertains to an antibody, or an antigen-binding fragment thereof, which selectively binds to Rin2 protein, or a portion of SEQ ID NO: 2 20 which is sufficient for Rin2 activity; in a particular embodiment, the antibody is a monoclonal antibody. The invention also relates to a method for assaying the presence of Rin2 protein in a cell, e.g., in a tissue sample, comprising contacting said cell with an antibody 25 which specifically binds to Rin2 protein.

The present invention also relates to an assay for identifying agents which alter the activity of Rin2 protein. For example, a cell population, e.g., a mast cell population, containing Rin2 protein can be 30 activated with a stimulus which activates at least one Ras-dependent pathway in the cell in the presence of an

-11-

agent to be tested, and the level of Rin2 activity can be assessed.

The invention further relates to methods of inhibiting the functional responses (e.g.,  
5 proliferation, functional activation) elicited by activation of Ras-dependent signaling pathways in cells, comprising contacting the cells with an agent which enhances or mimics the activity of Rin2 protein. The invention also relates to a method of treating a mammal  
10 in need thereof to inhibit functional responses elicited by activation of Ras-dependent signaling pathways, comprising administering to a mammal an agent which enhances or mimics the activity of native Rin2 protein.

The invention further relates to methods of inhibiting the functional responses elicited by Fc $\epsilon$ RI aggregation, particularly in mast cells, comprising contacting a mast cell population with an agent which enhances or mimics the activity of native Rin2 protein.

The invention relates to a method of treating a mammal  
20 in need thereof to inhibit the functional response elicited by Fc $\epsilon$ RI aggregation, comprising administering to a mammal an agent which enhances the activity of native Rin2 protein. For example, the invention relates to methods of inhibiting IgE and antigen-dependent  
25 release of mediators from mast cells. Such methods can be used to inhibit mediator release from mast cells and other effector cells that express Fc $\epsilon$ RI, such as basophils, monocytes/macrophages, dendritic cells, Langerhans' cells and eosinophils, thereby ameliorating  
30 disorders such as asthma and allergic diseases (e.g., hay fever and atopic eczema).

-12-

The invention further relates to methods of enhancing functional responses (e.g., proliferation, functional activation) elicited by activation of Ras-dependent signaling pathways in cells, comprising  
5 contacting the cells with an agent which inhibits the activity of Rin2 protein. The invention also relates to a method of treating a mammal in need thereof to enhance functional responses elicited by activation of Ras-dependent signaling pathways, comprising administering  
10 to a mammal an agent which inhibits the activity of Rin2 protein.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of the  
15 pathways of Ras activation and Ras-dependent changes in cell proliferation, differentiation and function.

Figure 2 is an illustration of alteration (increase or decrease) in Rin2 expression or activity and the corresponding effect on Ras-dependent signaling. Also  
20 illustrated are examples of instances in which it may be clinically desirable to alter Rin2 expression or activity and thereby alter, positively or negatively, Ras-dependent signaling.

Figure 3 is the cDNA sequence (SEQ ID NO: 1) and  
25 the predicted amino acid sequence (SEQ ID NO: 2) of the SY-A clone (Rin2). The predicted open reading frame begins at nucleotide 60 and ends at nucleotide 1535.

Figure 4 shows an alignment of the amino acid sequences of Rin2 with the GTPase binding homology (GBH)  
30 domains of Vps9p, Rin1, and JC265. The GBH domains of Vps9p, Rin1, and JC265 and the GBH motifs I, II, and III illustrated here represent those described in Burd et

-13-

al. (*Mol. Cell. Biol.* 16:2369-2377 (1996)). Proteins are identified on the left, and the position of the amino acid on each line is indicated. Identical amino acids or conservative substitutions present in three of 5 four proteins are shaded. Conservative substitutions for the alignment were assigned as follows: V, L, I, and M; K, R, and H; F, Y, and W; D and E; N and Q; S and T; and G and A.

Figure 5 is a graph of the kinetics of Erk-MAP kinase activation induced by Fc $\epsilon$ RI aggregation in C1.MC/C57.1 mast cells transfected with the control vector (pBK-CMV) or in C1.MC/C57.1 cells transfected with the antisense expression vector (pBK-CMV-SYA-AS).

Figure 6 is a graph of the release of  $^3$ H-serotonin induced by Fc $\epsilon$ RI aggregation in C1.MC/C57.1 mast cells transfected with the control vector (pBK-CMV) or in C1.MC/C57.1 cells transfected with the antisense expression vector (pBK-CMV-SYA-AS). Release of serotonin, assessed as percentage of specific release of 20  $[^3\text{H}]\text{-5HT}$ , was measured 10 minutes after challenge. An asterisk indicates  $P < 0.05$  versus corresponding values for cells transfected with the control pBK-CMV vector (n=4 to 5 per point).

Figures 7A and 7B are graphs of the kinetics of IL-6 release by mast cells. Figure 7A illustrates the kinetics of IL-6 release induced by Fc $\epsilon$ RI aggregation in C1.MC/C57.1 mast cells transfected with the control vector (pBK-CMV) or in C1.MC/C57.1 cells transfected with the antisense expression vector (pBK-CMV-SYA-AS). 30 Transfected mouse mast cells sensitized with anti-DNP IgE monoclonal antibody (mAb) for 2 hours were challenged with DNP-HSA. Figure 7B illustrates IL-6

-14-

release from transfected C1.MC/C57.1 mast cells 6 hours after stimulation by different concentrations of DNP-HSA. An asterisk indicates  $P < 0.05$  versus corresponding values for cells transfected with the 5 control pBK-CMV vector ( $n=4$  to 5 per point).

Figures 8A and 8B are graphs illustrating that expression of antisense *rin2* mRNA enhances cell proliferation in PC12 cells, a rat adrenal pheochromocytoma cell line. Rate of proliferation of 10 PC12 cells transfected with *rin2* antisense expression vector (pBK-CMV-SYA-AS), or PC12 cells transfected with the control CMV vector (pBK-CMV), or untransfected PC12 cells, was measured by assessing [<sup>3</sup>H]thymidine incorporation in cells which had been incubated with [<sup>3</sup>H] 15 thymidine for 3 hours after the cells had been cultured overnight in normal culture medium (Figure 8A), or in medium containing nerve growth factor (NGF) of indicated concentrations and 0.5% FCS (+NGF; Figure 8B). An asterisk indicates  $P < 0.01$  versus corresponding values 20 for untransfected PC12 cells or PC12 cells that had been transfected with the control pBK-CMV vector ( $n=4$  per point).

Figure 9 shows the orientation of overlapping human cDNA clones HRIN2-1B1 and HRIN2-2B1 relative to one 25 another.

Figures 10A-B show an alignment of murine Rin2 with the partial nucleotide sequence of human cDNA clone HRIN2-1B1 from nucleotide 51 through nucleotide 405 (SEQ ID NO: 12) and from nucleotide 885 through nucleotide 30 1144 (SEQ ID NO: 13). The sequence numbering is the nucleotide number in the murine Rin2 sequence.

-15-

Figures 11A-C show an alignment of murine Rin2 with the partial nucleotide sequence of human cDNA clone HRIN2-2B1 from nucleotide 532 to nucleotide 1276 (SEQ ID NO: 14) (Figures 11A-B) and a portion of the nucleotide sequence of the 3' end (SEQ ID NO: 15) (Figure 11C). In Figures 11A-B, the sequence numbering is the nucleotide number in the murine Rin2 sequence.

Figure 12 shows the interactions of murine Rin2 with murine H-Ras protein or yeast Ras2p protein in the yeast two-hybrid assay. Different segments of the murine Rin2 protein (shaded areas are those of greatest sequence homology to Vps9p, JC265 and Rin1; black areas are GBH motifs) were expressed as GAL4-activation domain fusion proteins, whereas the murine H-Ras and yeast Ras2p proteins were expressed as DNA-binding fusion proteins. Positive interactions were assessed with both a qualitative colony-lift  $\beta$ -galactosidase filter assay using X-gal as the substrate (colony color) and a quantitative liquid culture  $\beta$ -galactosidase assay using chlorophenol red-b-D-galactopyranoside (CPRG) as the substrate (relative  $\beta$ -gal units).

Figures 13A-B are graphs showing that *rin2* antisense transfectants release normal levels of preformed mediators but elevated levels of cytokines in response to Fc $\epsilon$ RI aggregation. Figure 13A shows the release of  $\beta$ -hexosaminidase ( $\beta$ -hex) or IL-6 induced by Fc $\epsilon$ RI aggregation in C1.MC/C57.1 mast cells transfected with the *rin2* antisense expression vector (AS) or the control CMV vector (control). Transfected cells sensitized with anti-DNP IgE mAb for 2 hours were challenged with DNP-HSA. Specific release of  $\beta$ -hexosaminidase (background release was  $\leq$  6%) was

-16-

measured 15 minutes after antigen challenge, whereas IL-6 release was measured by ELISA assay 6 hours after antigen challenge. The numbers over the columns indicate the numbers of stable lines of transfectants 5 established from two independent transfections and assayed in this experiment. An asterisk indicates  $p=0.03$  vs. control by unpaired Student's t test. Figure 13B shows the kinetics of IL-6 release induced by Fc $\epsilon$ RI aggregation in C1.MC/C57.1 mast cells transfected with 10 the antisense (AS) or control vector. An asterisk indicates  $p<0.01$  versus corresponding values for the control transfecant cells.

Figure 14 is a graph showing the kinetics of JNK activation induced by Fc $\epsilon$ RI aggregation in C1.MC/C57.1 15 cells transfected with the *rin2* antisense expression or the control vector. Findings are representative of those obtained with 3-4 different antisense and control transfecants.

Figure 15 is a graph showing the kinetics of p38 20 MAP kinase activation induced by Fc $\epsilon$ RI aggregation in C1.MC/C57.1 cells transfected with the *rin2* antisense expression vector or the control vector. Findings are representative of those obtained with 3-4 different antisense and control transfecants.

25

#### DETAILED DESCRIPTION OF THE INVENTION

In order to elucidate specific signaling pathways involved in Fc $\epsilon$ RI-mediated mast cell activation, the technique of mRNA differential display was used to 30 isolate cDNAs of genes that were differentially expressed in mast cells stimulated through the Fc $\epsilon$ RI. Work described herein identified the cDNA of a novel

-17-

gene, *rin2*, whose expression is rapidly increased in mammalian mast cells activated through Fc $\epsilon$ RI. The *rin2* gene product shares significant homology with members of a class of Ras-binding proteins, such as Rin1 and JC265  
5 (Colicelli et al., *Proc. Natl. Acad. Sci. USA* 88:2913-2917 (1991); Han and Colicelli, *Mol. Cell. Biol.* 15:1318-1323 (1995)), which have been shown to negatively regulate Ras-mediated signaling in *Saccharomyces (S.) cerevisiae*, and Vps9p (Burd et al.,  
10 *Mol. Cell. Biol.* 16:2369-2377 (1996)), which is required for vacuolar protein sorting. As shown herein, inhibition of Rin2 expression potentiates both the intracellular signaling responses and the cellular secretory responses that are induced by Fc $\epsilon$ RI  
15 aggregation. The work described herein supports the role of Rin2 as a novel negative regulator/effectector of Ras-dependent signaling pathways in mast cells.

The murine *rin2* gene encodes a protein of predicted molecular mass of 56.9 kDa which shares significant homology to a class of Ras-binding proteins represented by JC265, Rin1 and Vps9p. Rin1 and JC265 were identified by virtue of their ability to suppress an activated Ras mutant allele in *S. cerevisiae*, indicating that they can negatively regulate Ras-induced signaling  
25 responses (Colicelli et al., *Proc. Natl. Acad. Sci. USA* 88:2913-2917 (1991)). In addition, recent studies have shown that Rin1 interacts directly with the Ras protein in a manner similar to that observed with Raf1 and yeast adenylyl cyclase, which are known downstream effectors  
30 of Ras (Han and Colicelli, *Mol. Cell. Biol.* 15:1318-1323 (1995)). Thus, Rin1 binds primarily to the effector domain of Ras and functions as a downstream effector of

-18-

Ras-GTP. On the other hand, the gene encoding Vps9p was isolated in yeast by complementation of a vacuolar protein sorting defect associated with the *vps9* mutant (Burd et al., *Mol. Cell. Biol.* 16:2369-2377 (1996)).

5 Thus, Vps9p is a 53 kDa protein required for the proper sorting of several vacuolar proteins and may function as part of the vacuolar protein sorting machinery in *S. cerevisiae*.

The highest homology shared between JC265, Rin1, 10 Vps9p, and Rin2 is found in the GTPase binding homology (GBH) domain regions in which three putative GBH motifs have been described (Burd et al., *Mol. Cell. Biol.* 16:2369-2377 (1996)). In particular, the GBH motif II is situated within the regions of JC265 and Rin1 that 15 have been previously found to be shared with GAP and GAP-like proteins such as yeast IRA1 and IRA2, NF1, and sar1 (Colicelli et al., *Proc. Natl. Acad. Sci. USA* 88:2913-2917 (1991)). Furthermore, an FLP consensus motif can be detected within the GBH motif II regions of 20 JC265, Rin1, Vps9p, and Rin2, and this FLP motif aligns well with the FLR consensus motif (residues 901-903) present in the block 3A region that is common to all GAP proteins (Scheffzek et al., *Nature* 384:591-596 (1996)). Mutation analysis has shown that the phenylalanine 25 present at position 901 is required for core stabilization of the Ras protein and that leucine present at position 902 is important in the functional interaction between GAP and Ras (Miao et al., *J. Biol. Chem.* 271:15322-15329 (1996); Hettich and Marshall, 30 *Cancer Res.* 54:5438-5444 (1994); Brownbridge et al., *J. Biol. Chem.* 268:10914-10919 (1993)). The presence of the FLP consensus motif in the Rin2 amino acid sequence

-19-

provides additional support for the role of Rin2 as a regulator of Ras.

As also described herein, the SY-A (Rin2) clone has been used as a probe to screen a HT-29 human adenocarcinoma cDNA library. Two overlapping cDNA clones (HRIN2-1B1 (1.3 kb) and HRIN2-2B1 (2.9 kb)) have been identified which are highly homologous to that of the mouse Rin2 clone and which together represent the full-length human Rin2 (Rabex-5) cDNA sequence. The orientation of these overlapping clones is shown in Figure 9. The nucleotide sequence of each clone is shown in Figures 10A-B and 11A-C, respectively. The determination of the complete sequence of these clones can be carried out by the skilled artisan using methods well known in the art.

The invention pertains to an isolated nucleotide sequence encoding Rin2 protein. In one embodiment the isolated nucleotide sequence is a mammalian nucleotide sequence. In a particular embodiment the nucleotide sequence is a murine or human nucleotide sequence. As appropriate, nucleic acid molecules of the present invention can be RNA, for example, mRNA, or DNA, such as cDNA and genomic DNA. DNA molecules can be double-stranded or single-stranded; single stranded RNA or DNA can be either the coding (sense) strand or the non-coding (antisense) strand. The nucleotide sequence can encode a portion of the amino acid sequence of the Rin2 protein; alternatively, the nucleotide sequence can include at least a portion of the Rin2 amino acid coding sequence along with additional non-coding sequences, such as introns and non-coding 3' and 5' sequences (including regulatory sequences, for example).

-20-

Additionally, the nucleotide sequence can be fused to a marker sequence, for example, a sequence which encodes a polypeptide to assist in isolation or purification of the Rin2 protein. Such sequences include, but are not 5 limited to, those which encode a glutathione-S-transfereRase (GST) fusion protein and those which encode a hemagglutin A (HA) peptide marker from influenza.

As used herein, "isolated" is intended to mean that the material in question exists in a physical milieu 10 distinct from that in which it occurs in nature and/or has been completely or partially purified from other transcribed sequences (as in a cDNA or RNA library). Thus, an isolated gene or nucleotide sequence can include a gene or nucleotide sequence which is 15 synthesized chemically or by recombinant means.

Recombinant DNA contained in a vector is included in the definition of "isolated" as used herein. Also, isolated nucleotide sequences include recombinant DNA molecules in heterologous host cells, as well as partially or 20 substantially purified DNA molecules in solution. *In vivo* and *in vitro* RNA transcripts of the DNA molecules of the present invention are also encompassed by the term "isolated" nucleotide sequences. Such isolated nucleotide sequences are useful in the manufacture of 25 the encoded protein, as probes for isolating homologous sequences (e.g., from other mammalian species), for gene mapping (e.g., by *in situ* hybridization with chromosomes), or for detecting expression of the *rin2* gene in tissue (e.g., human tissue), such as by Northern 30 blot analysis.

The invention relates to nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO: 1 or

-21-

the complement of SEQ ID NO: 1. The invention also relates to nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO: 12 and/or SEQ ID NO: 13. That is, the nucleic acid molecule can comprise SEQ 5 ID NO: 12, SEQ ID NO: 13 or both SEQ ID NOS: 12 and 13. The invention further relates to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 14 and/or SEQ ID NO: 15. That is, the nucleic acid molecule can comprise SEQ ID NO: 14, SEQ ID NO: 15 or 10 both SEQ ID NOS: 14 and 15. In a particular embodiment, the nucleic acid molecule comprises one or more nucleotide sequences selected from the group consisting of SEQ ID NOS: 12, 13, 14 and 15.

The present invention also pertains to nucleotide sequences which are not necessarily found in nature but which encode Rin2 protein. Thus, DNA molecules which comprise a sequence which is different from the naturally-occurring nucleotide sequence but which, due to the degeneracy of the genetic code, encode Rin2 15 protein are the subject of this invention. The invention also encompasses variations of the nucleotide sequences of the invention, such as those encoding portions, analogues or derivatives of Rin2 protein. Such variations can be naturally-occurring, such as in 20 the case of allelic variation, or non-naturally-occurring, such as those induced by various mutagens and mutagenic processes. Included variations include, but are not limited to, addition, deletion and substitution 25 of one or more nucleotides which can result in conservative or non-conservative amino acid changes, including additions and deletions. Preferably, the nucleotide or amino acid variations are silent or 30

-22-

conserved, respectively; that is, they do not alter the characteristics or activity of Rin2 protein.

The invention described herein also relates to fragments of the isolated nucleic acid molecules 5 described above. The term "fragment" is intended to encompass a portion of a nucleotide sequence described herein which is from at least about 25 contiguous nucleotides to at least about 50 contiguous nucleotides or longer in length; such fragments are useful as 10 probes, e.g., for diagnostic methods and also as primers, and can encode functional portions of the Rin2 polypeptide. Particularly preferred primers and probes selectively hybridize to the nucleic acid molecule encoding Rin2 protein described herein. For example, 15 fragments which encode antigenic regions of the Rin2 protein described herein are useful.

The invention also pertains to nucleotide sequences which hybridize under medium stringency hybridization conditions (e.g., for selective hybridization) to a 20 nucleotide sequence described herein. Appropriate stringency conditions which are considered "medium stringency" are known to those skilled in the art or can be found in standard texts such as *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 25 6.3.1-6.3.6. Preferred hybridization conditions allow for specific hybridization of a nucleotide sequence to a nucleotide sequence described herein. Such hybridizable nucleotide sequences are useful as probes and primers for diagnostic applications.

30 The invention pertains to nucleotide sequences which have a substantial identity with the nucleotide sequence of SEQ ID NO: 1; particularly preferred are

-23-

nucleotide sequences which have at least about 75%, preferably at least about 80%, more preferably at least about 85%, and more preferably at least about 95% identity with nucleotide sequences described herein.

- 5 Particularly preferred in this instance are nucleotide sequences encoding polypeptides having at least one activity of the novel Rin2 protein described herein, or which hybridize with characteristic sequences of *rin2* and can therefore be used as probes or primers. For  
10 example, preferred nucleotide sequences encoding a polypeptide having the same or similar biological activity as the Rin2 protein, and nucleotide sequences encoding a polypeptide with the same or similar immunogenic or antigenic properties as the Rin2 protein  
15 are within the scope of the invention. As used herein, activities of the Rin2 protein include, but are not limited to, catalytic activity, binding function, antigenic function and oligomerization function.

This invention also pertains to an isolated protein  
20 or polypeptide which is a novel Rin2 protein, such as the protein or polypeptide encoded by any one or more of SEQ ID NOS: 1, 12, 13 and 14. Rin2 protein down-regulates the functional responses elicited by Fc $\epsilon$ RI aggregation in mast cells, including down-  
25 regulating the amounts of preformed mediator (serotonin) and cytokine (IL-6) released from these cells upon Fc $\epsilon$ RI-dependent stimulation. It may also down-regulate responses elicited at other types of receptors (such as TrkA receptors for NGF, c-kit receptors for SCF, etc.)  
30 in response to receptor interaction with ligand or due to auto-activation of the receptors (e.g., by activating mutations).

-24-

For example, an isolated protein of the invention may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs. In some instances, the isolated material will form part 5 of a composition (for example, a crude extract containing other substances), buffer system or reagent mix. In other circumstances, the material may be purified to essential homogeneity, for example as determined by PAGE or column chromatography such as 10 HPLC. According to the invention, the amino acid sequence of the polypeptide can be that of the naturally-occurring protein or can comprise alterations therein. Such alterations include conservative or non-conservative amino acid substitutions, additions and 15 deletions of one or more amino acids; however, such alterations should preserve at least one activity of the Rin2 protein, i.e., the altered or mutant protein should be an active derivative of the naturally-occurring protein. For example, the mutation(s) can preferably 20 preserve the three dimensional configuration of the binding and/or catalytic site of the native protein. The presence or absence of Rin2 activity can be determined by various functional assays, as described herein. Moreover, amino acids which are essential for 25 the function of the Rin2 protein can be identified by methods known in the art. Particularly useful methods include identification of conserved amino acids in the family or subfamily of Ras-interference or Ras-inhibiting proteins, site-directed mutagenesis and 30 alanine-scanning mutagenesis (for example, Cunningham and Wells, *Science* 244:1081-1085 (1989)), crystallization and nuclear magnetic resonance. The

-25-

altered polypeptides produced by these methods can be tested for particular biologic activities, including immunogenicity and antigenicity.

Specifically, appropriate amino acid alterations  
5 can be made on the basis of several criteria, including hydrophobicity, basic or acidic character, charge, polarity, size, the presence or absence of a functional group (e.g., -SH or a glycosylation site), and aromatic character. Assignment of various amino acids to similar  
10 groups based on the properties above will be readily apparent to the skilled artisan (see, for example, Figure 4); further appropriate amino acid changes can also be found in Bowie et al. (*Science* 247:1306-1310 (1990)).

15 The Rin2 polypeptide can also be a fusion protein comprising all or a portion of the Rin2 amino acid sequence fused to an additional component. Additional components, such as radioisotopes and antigenic tags, can be selected to assist in the isolation or  
20 purification of the polypeptide or to extend the half life of the polypeptide; for example, a hexahistidine tag permits ready purification by nickel chromatography.

Also included in the invention are novel polypeptides which are at least about 40% similar to the  
25 Rin2 protein described herein. However, polypeptides exhibiting lower levels of identity are also useful, particularly if they exhibit high, e.g., at least about 40%, similarity over one or more particular domains of the protein. For example, polypeptides sharing high degrees of identity or similarity over domains necessary  
30 for particular activities, including binding and enzymatic activity, are included herein. As used

-26-

herein, "similar" amino acids are intended to mean identical amino acids or conserved substitutions of amino acids.

Polypeptides described herein can be isolated from 5 naturally-occurring sources, chemically synthesized or recombinantly produced. Polypeptides or proteins of the present invention can be used as a molecular weight marker on SDS-PAGE gels or on molecular sieve gel filtration columns using art-recognized methods.

10 The invention also provides expression vectors containing a nucleic acid sequence encoding a polypeptide which is a Rin2 polypeptide, operably linked to at least one regulatory sequence. Many such vectors are commercially available, and other suitable vectors 15 can be readily prepared by the skilled artisan.

"Operably linked" is intended to meant that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleic acid sequence in a host cell. Regulatory sequences are art- 20 recognized and are selected to produce a polypeptide which is Rin2. Accordingly, the term "regulatory sequence" includes promoters, enhancers, and other expression control elements which are described in Goeddel, *Gene Expression Technology: Methods in Enzymology 185*, Academic Press, San Diego, CA (1990). 25 For example, the native regulatory sequences or regulatory sequences native to a transformed host cell can be employed. It should be understood that the design of the expression vector may depend on such 30 factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. For instance, the polypeptides of the present invention can

-27-

be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells or both (see, for example, Broach, et al., *Experimental Manipulation of Gene Expression*, ed. M. Inouye (Academic Press, 1983) p. 83; *Molecular Cloning: A Laboratory Manual*, 2nd Ed., ed. Sambrook et al. (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17). Typically, expression constructs will contain one or more selectable markers, including, but not limited to, the gene that encodes dihydrofolate reductase and the genes that confer resistance to geneticin, neomycin, tetracycline, ampicillin, chloramphenicol, kanamycin and streptomycin resistance.

15 Prokaryotic and eukaryotic host cells transfected by the described vectors are also provided by this invention. For instance, cells which can be transfected with the vectors of the present invention include, but are not limited to, bacterial cells such as *E. coli* (e.g., *E. coli* K12 strains), *Streptomyces*, *Pseudomonas*, *Serratia marcescens* and *Salmonella typhimurium*, insect cells (baculovirus), including *Drosophila*, fungal cells, such as yeast cells, plant cells and mammalian cells, such as mast cells, PC12 cells, EL-4 T cells or other T cells, Chinese hamster ovary cells (CHO) and COS cells.

Thus, a nucleotide sequence derived from the cloning of Rin2 described herein can be used to produce a recombinant form of the protein via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect,

-28-

plant or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other well known proteins. Similar procedures, or modifications thereof, can be employed to prepare recombinant proteins  
5 according to the present invention by microbial means or tissue-culture technology. Accordingly, the invention pertains to the production of Rin2 proteins or polypeptides by recombinant technology.

The proteins and polypeptides of the present  
10 invention can be isolated or purified (e.g., to homogeneity) from recombinant cell culture by a variety of processes. These include, but are not limited to, anion or cation exchange chromatography, ethanol precipitation, affinity chromatography and high  
15 performance liquid chromatography (HPLC). The particular method used will depend upon the properties of the polypeptide and the selection of the host cell; appropriate methods will be readily apparent to those skilled in the art.

20 The present invention also relates to antibodies which bind a polypeptide which is Rin2. For instance, polyclonal and monoclonal antibodies, including non-human and human antibodies, humanized antibodies, chimeric antibodies and antigen-binding fragments  
25 thereof (*Current Protocols in Immunology*, John Wiley & Sons, N.Y. (1994); EP Application 173,494 (Morrison); International Patent Application WO86/01533 (Neuberger); and U.S. Patent No. 5,225,539 (Winters)) which bind to the described protein are within the scope of the  
30 invention. A mammal, such as a mouse, rat, hamster or rabbit, can be immunized with an immunogenic form of Rin2 (e.g., Rin2 or a peptide comprising an antigenic

-29-

fragment of Rin2 which is capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. The protein or polypeptide can be administered in the presence of an adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibody.

Following immunization, anti-peptide antisera can be obtained, and if desired, polyclonal antibodies can be isolated from the serum. Monoclonal antibodies can also be produced by standard techniques which are well known in the art (Kohler and Milstein, *Nature* 256:495-497 (1975); Kozbar et al., *Immunology Today* 4:72 (1983); and Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)). The term "antibody" as used herein is intended to include fragments thereof, such as Fab and F(ab)<sub>2</sub>. Antibodies described herein can be used to inhibit the activity of Rin2 described herein, particularly *in vitro* and in cell extracts, using methods known in the art. Additionally, such antibodies, in conjunction with a label, such as a radioactive label, can be used to assay for the presence of the expressed protein in a cell from, e.g., a tissue sample, and can be used in an immunoabsorption process, such as an ELISA, to isolate the Rin2 protein. Tissue samples which can be assayed include human tissues, e.g., differentiated and non-differentiated cells. Examples include, but are not limited to, heart, lung, skin, skeletal muscle, bone marrow, thymus, lymph node,

-30-

spleen, kidney, liver, brain, pancreas, gastrointestinal tract, gonads (testes, ovaries), fibroblasts and epithelium.

The present invention also pertains to pharmaceutical compositions comprising polypeptides described herein. For instance, a polypeptide or protein, or prodrug thereof, of the present invention can be formulated with a physiologically acceptable medium to prepare a pharmaceutical composition. The particular physiological medium may include, but is not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to well known procedures, and will depend on the ultimate pharmaceutical formulation desired. Methods of introduction of exogenous peptides at the site of treatment include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, intranasal, intrarectal, intravaginal, and aerosol (for administration to the respiratory tract). Other suitable methods of introduction can also include gene therapy, rechargeable or biodegradable devices and slow release polymeric devices. The pharmaceutical compositions of this invention can also be administered as part of a combinatorial therapy with other agents.

While the intracellular events which lead to the activation of cell proliferation, differentiation, or functional responses (e.g., Fc $\epsilon$ RI-dependent mast cell mediator secretion) have been investigated in great

detail, equally as interesting are the pathways which can suppress such responses. For example, recent evidence indicates that certain cell surface receptors, such as Fc $\gamma$ RIIb or gp149 in mast cells, can mediate signals which can down-regulate the cell activation signals which are transduced by other cell surface receptors, including the mast cell Fc $\epsilon$ RI (Scharenberg and Kinet, *Cell* 87:961-964 (1996)). However, the work described herein is directed to searching for intracellular molecules which function to down-regulate the signaling pathways in which Ras plays a role, including those which govern the secretion of mast cell mediators or the proliferative responses of neuronal cells or other cell types. Ras plays a pivotal role in signaling pathways which mediate diverse cellular responses to a spectrum of receptor-ligand interactions (Bourne et al., *Nature* 348:125-132 (1990); Marshall, *FASEB J.* 9:1311-1318 (1995)). Recent studies have shown that the functional activation of mast cells which is induced by the aggregation of Fc $\epsilon$ RI by IgE and specific antigen is associated with the activation of Ras and its effector pathways (Beaven and Baumgartner, *Curr. Opin. Immunol.* 8:766-772 (1996); Jabril-Cuenod et al., *J. Biol. Chem.* 271:16268-16272 (1996)).

Fc $\epsilon$ RI-mediated Ras activation in mast cells is accompanied by activation of the Shc-Grb2-Sos pathway, which precedes Ras activation, as well as the Ras effector pathway of Raf-1/Erk-MAP kinases (Jabril-Cuenod et al., *J. Biol. Chem.* 271:16268-16272 (1996)). Recent studies with rat RBL-2H3 mast cells have demonstrated that Fc $\epsilon$ RI-induced Ras activation results in a transcription activation mediated by Elk-1 and NFAT

-32-

(Turner and Cantrell, *J. Exp. Med.* 185:43-53 (1997)). The Elk-1 transcription factor exerts its effects by interacting with the serum response element, which is a regulatory component of early response genes such as 5 *c-fos* (Treisman, *Curr. Opin. Genet. Dev.* 4:96-101 (1994)), whereas members of the NFAT transcription factor family form regulatory complexes with inducible nuclear factor such as Fos or Jun (Rao, *Immunol. Today* 15:274-281 (1994)). Such complexes interact with 10 cytokine gene promoters and activate transcriptions of genes for IL-2, IL-4, GM-CSF, and TNF- $\alpha$  (Rao, *Immunol. Today* 15:274-281 (1994)). In mast cells, NFAT is implicated in the regulation of transcription of the IL-4 gene (Weiss et al., *Mol. Cell. Biol.* 16:228-235 15 (1996)). Interestingly, the activation of the Raf-1/MEK/Erk cascade is necessary and sufficient for the activation of Elk-1 in mast cells, whereas the induction of NFAT by Fc $\epsilon$ RI stimulation requires the activity of Rac-1 (Turner and Cantrell, *J. Exp. Med.* 20 185:43-53 (1997)). Earlier studies have shown that Rin1 interacts directly with Ras in a manner that is characteristic of Raf-1, suggesting that Rin1 is a negative downstream effector of Ras (Han and Colicelli, *Mol. Cell. Biol.* 15:1318-1323 (1995)). 25 As shown herein, reduced expression of Rin2 can dramatically potentiate the downstream signaling responses mediated by Ras. In mouse mast cells transfected with the *rin2* antisense expression vector, the induction of early-response gene expression and the 30 activation of MAP kinase activity in response to Fc $\epsilon$ RI crosslinking are significantly augmented, as compared with those observed in mast cells transfected with a

-33-

control vector. These results support a role of Rin2 as a negative effector/regulator of Ras. Furthermore, reduced expression of Rin2 can markedly enhance the functional responses elicited by Fc $\epsilon$ RI aggregation in 5 mouse mast cells; the expression of antisense *rin2* in mouse mast cells can significantly increase the amounts of preformed mediators (e.g., 5-HT) and cytokine (e.g., IL-6) released from these cells upon Fc $\epsilon$ RI-dependent stimulation. These data indicate that Rin2 can 10 negatively regulate the Ras-mediated signaling responses in mast cells that lead to both the release of preformed mediators and the transcription of cytokine genes and the secretion of the corresponding products.

Stimulation of mast cells by IgE and antigen results in 15 Ras activation followed by a rapid and transient induction of *rin2* mRNA expression. Rin2 appears to exert its effects by down-regulating the signaling and functional responses elicited by Fc $\epsilon$ RI aggregation. Thus, during the period subsequent to mast cell 20 activation, Rin2 may function as a cellular switch in turning off ongoing signaling initiated by Ras activation. In mast cells, such a regulatory process may be important as a feedback mechanism to restore the perturbed cellular environment to its normal basal 25 physiological state after the cessation of cell activation.

Furthermore, *rin2* mRNA expression is significantly elevated in mouse mast cells stimulated with stem cell factor (SCF), with a kinetics of activation that is very 30 similar to that which is observed in mast cells after Fc $\epsilon$ RI-dependent stimulation. SCF, the cognate ligand for the c-kit tyrosine kinase receptor, is a major mast

- 34 -

cell growth factor which has been shown to be critical in the maturation and development/function of rodent and human mast cells (Galli et al., *Adv. Immunol.* 55:1-96 (1994)). The interaction of SCF with the c-kit tyrosine kinase receptor has been shown to induce an activation of Ras and its effector pathways via the activation of the Shc-Grb2-Sos signaling pathway (Duronio et al., *Proc. Natl. Acad. Sci. USA* 89:1587-1591 (1992); Tauchi et al., *J. Exp. Med.* 179:167-175 (1994)).

As shown herein, nerve growth factor (NGF) can also elicit a rapid and sustained induction of expression of *rin2* mRNA transcript through its receptor. NGF has been shown to induce neuronal differentiation and cessation of cell proliferation in PC12 cells via its interaction with TrkA receptor tyrosine kinase (Barbacid, *J. Neurobiol.* 25:1386-1403 (1994); Bothwell, *Ann. Rev. Neurosci.* 18:223-253 (1995)). PC12 cells are a commonly used *in vitro* model of neuronal development and function (Greene and Tischler, *Proc. Natl. Acad. Sci. USA* 73:2424-2428 (1976)). Previous studies have indicated that NGF stimulation of TrkA receptors in PC12 cells leads to Ras activation and the activation of the Raf-1/MEK/MAP kinases effector pathway (Thomas et al., *Cell* 68:1031-1040 (1992); Wood et al., *Cell* 68:1041-50 (1992)). Results presented herein show that PC12 cells which have been transfected with the pBK-CMV-SYA-AS vector and therefore have reduced expression of Rin2 can still differentiate in response to NGF stimulation. However, these transfected cells exhibit a higher rate of cell proliferation than that detected in control PC12 cells. This effect is observed in both unstimulated transfected PC12 cells and transfected PC12 cells

-35-

stimulated with NGF. NGF is also known to have a transient proliferative effect on PC12 cells, which precedes the better known anti-mitogenic effects of NGF on these cells. Thus, by suppressing Ras-dependent  
5 proliferative effects, Rin2 may play a role in mediating the anti-mitogenic effects of NGF on these neuronal cells. Taken together, these data indicate that the expression of the *rin2* gene transcript can be differentially regulated in mast cells and in PC12 cells  
10 via different ligand-receptor interactions.

As disclosed herein, activation of a mouse T cell lymphoma line (EL-4 cells) via the T cell receptor, by stimulating the cells with antibodies directed against CD3 and CD28, also elicits a rapid and transient  
15 induction of expression of *rin2* mRNA transcript. T cells are essential for the expression of an adequate immune response, but inappropriate activation of T cells is thought to contribute to a wide variety of diseases with "autoimmune" components, such as autoimmune (Type  
20 I) diabetes mellitus, rheumatoid arthritis, ankylosing spondylitis, sarcoidosis, Sjögren's syndrome, multiple sclerosis, inflammatory bowel disease (i.e., Crohn's disease and ulcerative colitis), dermatomyositis, scleroderma, polymyositis, systemic lupus erythematosus,  
25 biliary cirrhosis, autoimmune thyroiditis, and autoimmune hepatitis, as well as many dermatological disorders, including psoriasis, contact sensitivity and atopic dermatitis (Schwartz, Autoimmunity and Autoimmune Diseases, in *Fundamental Immunology*, Third Edition,  
30 Paul, ed. (Raven Press, NY, pp. 1033-1097 (1993)); Rich, *Clinical Immunology: Principles and Practice*, Mosby, St. Louis (1996)). T cells are also important in the

-36-

process of tissue graft rejection, such as grafts of bone marrow, hematopoietic stem cells, skin, heart, lung, liver, kidney, pancreas and intestine, among others, and in graft-versus-host disease; T cells can 5 also undergo neoplastic transformation, resulting in the development of leukemias or lymphomas. The findings with EL-4 T cells disclosed herein support the role of Rin2 as a negative Ras effector/regulator mediating diverse cellular responses in T cells. Taken together 10 with the results disclosed herein regarding the role of Rin2 in regulating cellular responses in mast cells and PC12 adrenal pheochromocytoma cells, the findings in EL-4 T cells are also consistent with the hypothesis that Rin2 is a negative Ras effector/regulator mediating 15 diverse functional responses in different cell types. There are likely to be many other cell types in which Rin2 may down-regulate Ras-dependent functional responses, since Ras is involved in influencing functional responses in diverse cell types (Marshall, 20 *FASEB J* 9:1311-1318 (1995)). Additionally, cells which express the Fc $\epsilon$ RI receptor, T cell receptor, TrkA and c-kit, as well as diverse other receptors which signal by mechanisms which are influenced by Ras, can have their 25 Ras-dependent functional responses negatively regulated by Rin2.

It will be apparent to the skilled artisan that the identification of the novel protein Rin2, which can inhibit Ras-dependent cellular signaling, has many commercial applications. The Ras effector signaling 30 pathways have been shown to be important in mediating signal transduction from the cell membrane to the

nucleus in many oncogenic, mitogenic, and developmental processes.

As a result of Rin2's ability to inhibit IgE and antigen-dependent release of mediators from mast cells, 5 compositions which can enhance Rin2 expression and/or function (e.g., Rin2 itself or other compounds), or which mimic these actions (e.g., molecules synthesized based on the structure/functional characteristics of the Rin2 protein), can be used to inhibit mediator release 10 from effector cells in disorders such as asthma and other allergic diseases (e.g., hay fever and atopic eczema), thus ameliorating these disorders.

The ability of Rin2 to negatively regulate Ras-dependent signal transduction in other cell types 15 (in addition to mast cells) suggests that compounds (e.g., isolated Rin2, Rin2 "mimics" or agents that can enhance expression of native Rin2) that are able to block such Ras-dependent cellular signaling pathways can produce clinically desirable effects. Mutated forms of 20 cellular Ras genes, resulting in oncogenic activation of Ras proteins, have been found to be among the most common genetic abnormalities associated with human cancer. The early stage in multistage tumor progression has been associated with Ras activation, and disruption 25 of the oncogenic Ras genes in colon cancer cell lines has been shown to revert the cells to a more regulated state of growth (Shirasawa et al., *Science* 260:85-88). Thus, the *rin2* cDNA clone can be used to identify compounds that can block Ras-induced cell transformation 30 *in vitro*. These compounds can include isolated Rin2 (e.g., overexpression of Rin2), derivatives (modified forms) of Rin2, and compounds identified by another

-38-

method, e.g., computer modeling of compounds designed with reference to structures and/or characteristics of Rin2 and synthesized by known methods (e.g., chemical synthesis, peptide chemistry). These compounds can be  
5 tested for their ability to function as Ras-specific anti-neoplastic agents for the treatment of cancer, or as Ras-specific agents which inhibit other examples of clinically unwanted, but non-neoplastic, Ras-dependent cellular proliferation.

10 Mutations in the genes encoding other molecules which are involved in Ras-dependent intracellular signaling have been linked to human diseases with a genetic component, such as some cases of melanoma. The *rin2* cDNA clone can be used to map, identify and isolate  
15 or clone the human counterpart of the *rin2* gene and, ultimately, to assess whether mutations of Rin2 and/or genes identified using Rin2 are linked to known human diseases.

The *rin2* cDNA clone can be used in a yeast  
20 two-hybrid system to identify other novel cellular proteins that interact with Rin2 and/or Ras and thus regulate the signaling responses along Ras effector pathways. Using a yeast two-hybrid system (Fields and Sternglanz, *Trends Genet.* 10:286-292 (1994)), specific  
25 regions of the Rin2 protein that interact with Ras can be identified. The elucidation of the mechanism of protein-protein interaction between Rin2 and Ras will be useful in the development of small molecule drugs that interact specifically with Ras and inhibit or enhance  
30 its function.

Using the *rin2* cDNA clone, the Rin2 protein can be produced using a protein expression system. The

-39-

partially purified Rin2 protein, or a synthetic peptide based on the known Rin2 amino acid sequence, can be used to raise Rin2-specific antibodies which can be utilized as laboratory reagents for studying the Ras effector pathways, as well as "humanized" antibodies for potential clinical applications. Rin2 is an intracellular protein. Thus, small fragments of antibodies against Rin2, perhaps specifically modified to enhance their ability to identify specific cell types and to pass through the cells' surface (plasma membrane), may be required to gain appropriate access to the intracellular Rin2 target.

However, other natural or newly-synthesized chemicals that, because of their properties, can readily enter cells and achieve intracellular concentrations which are sufficient to permit the expansion of the desired interactions with Rin2, can be identified via their ability to interact with Rin2, Rin2 fragments or Rin2-mimicking compounds, in various *in vitro* systems. Rin2-mimicking compounds are defined herein as peptide or protein compounds which may be unrelated to Rin2 in amino acid sequence, or non-peptide based compounds, which express sufficient structural similarity to native Rin2 or fragments thereof to permit such compounds to be used for initial screening of compounds, including those generated through recombinatorial chemistry approaches, to identify those which can interact with active Rin2 sufficiently to enhance or inhibit its function.

As used herein, "inhibition" is intended to encompass any reduction in the quality or quantity of the assessed activity, including complete abolishment thereof. The term "enhancement", as used herein is

-40-

intended to mean any increase in the quality or quantity of the assessed activity, including increases resulting from mimicking of an assessed activity. As used herein, "alteration" is intended to mean either inhibition or 5 enhancement of the quality or quantity of the assessed activity. As used herein, "mimicking" is intended to mean that the assessed activity is substantially similar to the corresponding activity of Rin2.

Depending on the circumstances, it may be desirable 10 either to inhibit or to enhance Rin2 activity, in order to enhance or to suppress, respectively important Ras-dependent signaling pathways. Several examples of the inhibition of Rin2 activity, resulting in reduced Rin2-dependent inhibition of Ras-dependent pathways, with 15 subsequent enhancement of such pathways, can be envisioned. Enhancement of Ras-dependent pathways promoting the proliferation and functional activation of cells involved in wound healing, including the development of new blood vessels (i.e., angiogenesis) in 20 areas of ischemic tissue (e.g., in tissues such as cardiac or skeletal muscle, which are ischemic because of the consequences of arteriosclerosis), can have great therapeutic benefit. Enhancement of the Ras-dependent pathways which contribute to the proliferation of, and 25 production of mediators and cytokines by, T cells and mast cells, and other cell types activated through the same or distinct receptors, may be of great benefit in diseases that are associated with diminished numbers and/or function of these cells, such as in patients 30 infected with the human immunodeficiency virus (HIV), or receiving treatment with radiation or chemical agents for the treatment of cancer. Enhancement of Ras-

-41-

dependent signaling in neuronal cells in response to NGF or other neurotrophic factors may be desirable in settings characterized by neuronal loss, or when neural regeneration is an objective (Satoh et al., *Mol. Cell. Biol.* 7:4553-4556 (1987); Szeber'nyi et al., *Mol. Cell. Biol.* 10:5324-5332 (1990); Qiu and Green, *Neuron* 7:937-946 (1991); Muroya et al., *Oncogene* 7:277-281 (1992)). For example, Ras-dependent pathways, such as the functional responses elicited by Fc $\epsilon$ RI aggregation, can 10 be inhibited by enhancing the activity of Rin2. Agents which enhance Rin2 activity include, but are not limited to, isolated Rin2, Rin2 mimics or agents that can enhance expression of native Rin2.

In contrast, enhancement of Rin2 activity, 15 resulting in increased Rin2-dependent inhibition of Ras-dependent pathways, with subsequent inhibition of such pathways, can also be useful. For example, it may be desirable to inhibit TCR- and Ras-dependent pathways promoting the proliferation and functional activation of 20 cells involved in tissue graft rejection, graft-versus-host disease, and autoimmune disorders or T cell-associated disorders, e.g., autoimmune (Type I) diabetes mellitus, rheumatoid arthritis, ankylosing spondylitis, sarcoidosis, Sjögren's syndrome, multiple sclerosis, 25 inflammatory bowel disease (i.e., Crohn's disease and ulcerative colitis), dermatomyositis, scleroderma, polymyositis, systemic lupus erythematosus, biliary cirrhosis, autoimmune thyroiditis, and autoimmune hepatitis, as well as many dermatological disorders, 30 including psoriasis, contact sensitivity and atopic dermatitis. In asthma and allergic diseases such as hay fever and atopic dermatitis, it is desirable to inhibit

-42-

the Ras-dependent functional responses induced by Fc $\epsilon$ RI aggregation in cells bearing the Fc $\epsilon$ RI receptor, e.g., mast cells. Specifically, the intracellular signaling (e.g., activation of MAP kinase) and cellular secretory 5 responses (e.g., release of preformed mediators, such as 5-HT, and cytokines, such as IL-6) can be inhibited by enhancing Rin2 activity. Additionally, enhancement of Rin2 activity can also be used to inhibit cellular proliferation through the TrkA receptor, e.g., in PC12 10 cells, and to inhibit Ras-dependent cellular proliferation, including cancers or other unwanted neoplastic or non-neoplastic proliferation of cells.

Alteration of Rin2 activity can be accomplished by increasing or decreasing the amount of Rin2 protein 15 present to inhibit or enhance, respectively, Ras-dependent pathways, the activity of the Rin2 protein, or both. For example, the activity of the Rin2 protein described herein can be enhanced by overexpressing isolated Rin2 protein from an appropriate expression 20 construct in a host cell. Alternatively, a cell population of interest can be treated with one or more agents, such as SCF, that enhance expression or activity of native Rin2 present in the cell population.

Additionally, compounds which mimic Rin2 activity are 25 useful in applications in which native or isolated Rin2 are useful. Useful compounds will be able to mimic at least one appropriate activity of Rin2, e.g., binding activity, catalytic activity, and include fragments and derivatives of Rin2. Alternatively, Ras-dependent 30 pathways can be enhanced by inhibiting the activity of Rin2. Agents which inhibit Rin2 activity include, but

-43-

are not limited to, agents that can inhibit expression of native Rin2 and agents which bind native Rin2.

The invention also pertains to methods of inhibiting functional responses induced by Ras-dependent 5 signaling pathways in a mammal by administering to the mammal an effective amount of an agent which enhances the activity of Rin2. The invention also pertains to methods of inhibiting cellular proliferation in a mammal by administering to the mammal an effective amount of an 10 agent which enhances the activity of Rin2. The invention also encompasses methods of inhibiting Ras-dependent cellular proliferation, including cancers, neoplastic and non-neoplastic proliferation, in a mammal by administering to the mammal an effective amount of an 15 agent which enhances the activity of Rin2.

The invention also pertains to methods of enhancing functional responses induced by Ras-dependent signaling pathways in a mammal by administering to the mammal an effective amount of an agent which inhibits the activity 20 of Rin2.

The present invention also relates to an assay for identifying agents which alter the activity of Rin2, such as agents which enhance or inhibit the activity of Rin2. For example, a cell containing the Rin2 protein, 25 or an active fragment or derivative thereof, can be activated via a specific receptor that induces signaling that is modulated by Rin2, in the presence of an agent to be tested, and the level of Rin2 activity can be assessed; in a preferred embodiment, the level of Rin2 30 activity can be assessed and compared with the corresponding level in a control in the absence of the agent to be tested. The activity of Rin2 protein can be

-44-

- assessed directly, e.g., by assessing the expression levels of the protein, or indirectly, such as by assessing functional responses e.g., activation of MAP kinase activity or secretion of 5-HT or IL-6. The cell
- 5 containing Rin2, or a derivative or portion thereof having Rin2 activity, can be contacted directly or indirectly with the stimulus in the presence of the agent to be tested. Enhancement of Rin2 activity, or an increase in the level of Rin2 activity relative to a
- 10 control, indicates that the agent is an agonist of Rin2 activity. Similarly, inhibition of Rin2 activity, or a decrease in the level of Rin2 activity relative to a control, indicates that the agent is an antagonist of Rin2 activity.
- 15 The present invention also relates to novel agents identified by the assay described above. Agents identified by the assay described herein may enhance (e.g., prolong or increase) or inhibit (e.g., shorten or decrease) the activity of the Rin2 protein.
- 20 The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all references cited herein are hereby incorporated herein by reference.

-45-

## EXAMPLES

### MATERIALS AND METHODS

#### Cytokines, cDNA Probes and Antibodies

Recombinant mouse IL-3 was purchased from BioSource International (Camarillo, CA). The c-fos probe is a 1.2 kb HindIII/EcoRI mouse cDNA fragment generously provided by Dr. Brent Cochran. The cDNA probes were labeled with [ $\alpha$ -<sup>32</sup>P] dCTP (6000 mCi/mmol) (New England Nuclear, Boston, MA) using the exo(-) Klenow fragment of DNA polymerase I with random nonamer primers (Stratagene, La Jolla, CA). Purified mouse 2.5 S NGF was purchased from Upstate Biotechnology (Lake Placid, NY). For T-cell activation experiments, purified hamster anti-mouse CD3e and anti-mouse CD28 monoclonal antibodies were purchased from Pharmingen (San Diego, CA).

#### Cell Culture

Bone marrow-derived cultured mast cells (BMCMCs) were obtained by maintaining the femoral bone marrow cells of 4-6 week-old BALB/c mice in suspension in IL-3-containing conditioned medium, consisting of 10% heat-inactivated fetal calf serum (FCS) (Sigma Chemical Company, St. Louis, MO), 5 x 10<sup>-5</sup> M 2-mercaptoethanol (Sigma) and 2 mM L-glutamine (GIBCO Laboratories, Grand Island, NY) in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO Laboratories, Grand Island, NY) (complete medium) supplemented with 20% (v/v) of either supernatants from Concanavalin A-activated spleen cells or WEHI-3 cell-conditioned medium (Nabel et al., *Nature* 291:332-334 (1981); Galli et al., *J. Cell Biol.* 95:435-444 (1982)). The cells were resuspended in fresh

-46-

conditioned medium 1-2 times a week. After 4-5 weeks, at least 95% of cells that remained in the cultures were identifiable as mast cells, as determined by neutral red or May-Grünwald/Giemsa staining. C1.MC/C57.1 cells, a 5 cloned growth factor-independent mouse mast cell line of BALB/c origin (Young et al., *Proc. Natl. Acad. Sci. USA* 84:9175-9179 (1987); Tsai et al., *FASEB J.* 10, Abstract A1268 (1996)), were maintained in complete medium lacking exogenous growth factors. PC12 cells, a rat 10 adrenal pheochromocytoma cell line, were obtained from American Type Culture Collection (ATCC No. CRL1721). They were grown in RPMI 1640 medium supplemented with 10% horse serum (HS) and 5% FCS. EL-4 cells, a mouse lymphoma T cell line, were obtained from ATCC (ATCC No. 15 TIB39) and grown in DMEM medium supplemented with 10% HS.

#### Fc $\epsilon$ RI Cross-Linking

The IgE anti-DNP mAb-producing hybridoma 20 H1-DNP-e-26 (Liu et al., *J. Immunol.* 124:2728-2737 (1980)), generously provided by Drs. F.-T. Liu and D.H. Katz, was used to generate ascites. BMCMC or C1.MC/C57.1 mast cells were sensitized with the ascites diluted 500-fold in complete medium (final IgE 25 concentration of approximately 8  $\mu$ g/ml) for 2 hours at 37°C, washed with medium, then resuspended with medium containing DNP<sub>30-40</sub>-HSA (Sigma) at 50 ng/ml at 37°C. These conditions of sensitization with IgE and stimulation with specific antigen have been shown to be 30 adequate for the activation of such cells for [<sup>3</sup>H] 5-HT

-47-

release (Gordon and Galli, *J. Exp. Med.* 174:103-107 (1991)).

#### mRNA Differential Display

5        Differential display was performed using the RNAmap KitA (GenHunter, Nashville, TN) according to the manufacturer's specifications. Specifically, total RNA was extracted from cultured cells using the RNAzol B method (Biotecx Laboratories, Houston, TX) and  
10 subsequently digested with DNaseI (1 unit/10 µg) (Promega, Madison, WI) for 30 minutes at 37°C. Approximately 2 µg of digested RNA was subjected to the reverse transcription reaction with one of the four T<sub>12</sub>MN anchored primers (N=G, A, T, or C) at 65°C for 5  
15 minutes, at 37°C for 60 minutes, and then at 95°C for 5 minutes. The MMLV reverse transcriptase (100 units) was added after 10 minutes of incubation at 37°C. The resulting cDNAs were further amplified by PCR (final volume, 20 µl) with GeneAmp 1XPCR Buffer (Perkin Elmer,  
20 Foster City, CA), 2 µM dNTPs, 0.2 µM AP-primers (AP-1:  
5'-AGCCAGCGAA-3' (SEQ ID NO: 3); AP-2: 5'-GACCGCTTGT-3'  
(SEQ ID NO: 4); AP-3: 5'-AGGTGACCGT-3' (SEQ ID NO: 5);  
AP-4: 5'-GGTACTCCAC-3' (SEQ ID NO: 6); and AP-5:  
5'-GTTGCGATCC-3' (SEQ ID NO: 7)), 1 µM T<sub>12</sub>MN primer (the  
25 same primer used in the respective reverse transcription reaction), 2 µl reverse transcription mix, 1 µl <sup>35</sup>S-dATP (1200 Ci/mmol) (NEN, Boston, MA), and 1 unit AmpliTaq DNA polymerase (Perkin Elmer) at 94°C for 30 seconds, 40°C for 2 minutes, and 72°C for 30 seconds for 40  
30 cycles, followed by an additional 5 minutes at 72°C. 3.5 µl of each sample was run on 6% sequencing gels.

-48-

Differentially-expressed PCR products were excised from the gel, extracted with water, and reamplified by PCR using the same set of primers. The reamplified PCR segments were <sup>32</sup>P-labeled as probes for Northern blot analysis, or subcloned into pGEM-T Vector (Promega) and sequenced using Sequenase version 2.0 (U.S. Biochemical, Cleveland, OH).

#### **Northern Hybridization**

10 Total RNA was prepared from cultured cells or from mouse tissues using the RNAzol B method (Biotecx) according to the manufacturer's specifications; 10 or 15 µg of total RNA were loaded in each lane and electrophoresed in 1% agarose-formaldehyde denaturing 15 gel, and then transferred onto Zetabind nylon membrane (Cuno, Meriden, CT). RNA blots were hybridized at 42°C for 16-18 hours with 10<sup>6</sup> cpm/ml of <sup>32</sup>P-labeled cDNA probes, washed at 42°C to a final stringency of 0.2X SSC, then exposed to Kodak XAR-5 films at -80°C.

20

#### **cDNA Library Screening**

The 60-4 probe isolated from the differential display studies (see Results) was radiolabeled and used as a probe to screen a BMCMC cDNA library, which had 25 been constructed in our laboratory using the Uni-ZAP XR Vector (Stratagene, La Jolla, CA). One positive clone, SY-6, with 1.1 kb insert was isolated. To further isolate the full-length cDNA, the SY-6 clone was used as a probe to screen a mouse brain cDNA library in Uni-ZAP 30 XR Vector purchased from Stratagene. Both libraries were screened by filter replica hybridization, and

-49-

plaque-purified phages were isolated with three rounds of screening. *In vivo* excision of plasmids from the lamda vector was performed using ExAssist helper phage and SOLR recipient cells (Strategene).

5

#### ***In Vitro Transcription/Translation***

Approximately 1  $\mu$ g of the pBluescript SK (-) containing the full-length SY-A cDNA clone was transcribed and translated *in vitro* using the TNT T,

- 10 Coupled Reticulocyte Lysate System (Promega). Proteins were labeled in the translation step with 40  $\mu$ Ci of [ $^{35}$ S] methionine (1,000 Ci/mmol) (Amersham, Arlington, IL), run on an 8% SDS/PAGE, and visualized by autoradiography.

15

#### **Rin2 Antibody and Western Blotting**

Anti-Rin2 antibodies were prepared by immunizing rabbits with a synthetic peptide corresponding to the 16 N-terminal residues of murine Rin2 (KSERRGIHVDQSELLC; SEQ ID NO: 16) conjugated to keyhole limpet hemacyanin.

- 20 The antibody was affinity-purified on a peptide column using a cysteine residue of the peptide coupled to an iodoacetamide on Sepharose beads. Cells were lysed in lysis buffer (20 mM Tris 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerolphosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml leupeptin, 1 mM PMSF). Resulting cell lysates were subjected to 12% SDS-PAGE and electroblotted. Membranes were incubated with the anti-Rin2 antibody (1:500), and the antigen-30 antibody complexes were visualized with anti-rabbit IgG

-50-

secondary antibody using enhanced chemiluminescence (ECL).

**Yeast Two-Hybrid Assay**

5       The protein-protein interactions between murine Rin2 and the murine or yeast Ras protein were assessed using the MATCHMAKER Two-Hybrid System1 (Clontech) according to the instructions supplied by the manufacturer. The entire open reading frame, or  
10 segments of the open reading frame of the SY-A clone was subcloned, in frame, into the pACT2 vector. The murine H-Ras cDNA was generated from total RNA of C1.MC/C57.1 by RT-PCR using the 5' primer  
GCGGAATTCATGACAGAATAACAAGCTTGTG (SEQ ID NO: 17) and the  
15 3' primer GACGGATCCCTCAGGACAGCACACACTTGC (SEQ ID NO: 18) and subcloned into pAS2-1. The yeast Ras2p cDNA was generated from *S. cerevisiae* poly A+ RNA (Clontech) by RT-PCR using the 5' primer CGATGTCGACCATGCCTTGAAACAAGTCG (SEQ ID NO: 19) and the 3' primer  
20 GATAGGATCCACCCGATCCGCTTTG (SEQ ID NO: 20) and subcloned into pAS2-1. A Cys-to-Ser mutation at residues 318 of Ras2p, which suppresses palmitoylation, was constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Y187 yeast strain was cotransformed with  
25 the constructs described above and the transformants were selected on -Leu/-Trp plates.  $\beta$ -galactosidase activity was determined for colonies that appeared after 3-4 days by a qualitative colony-lift filter assay using X-gal as substrate and a quantitative liquid culture  
30 assay using CPRG as substrate.

-51-

#### Generation of Stable Transfectants

The SY-A cDNA clone was digested with XbaI and XhoI restriction enzymes, and the entire full-length cDNA insert released was ligated to the pBK-CMV expression vector (Stratagene) to generate the *rin2* antisense expression plasmid, pBK-CMV-SYAS. Plasmid pBK-CMV-SYAS and the vector control plasmid pBK-CMV were transfected into C1.MC/C57.1 mast cells or PC12 cells using LIPOFECTAMINE Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. For C1.MC/C57.1 cells, transfected cells were selected in Geneticin (G418) (Life Technologies) at 2 mg/ml for 10 days and then at 0.8 mg/ml thereafter. G418-resistant mast cell colonies were observed approximately two weeks after transfection. For PC12 cells, transfected cells were selected in 0.6 mg/ml G418 and G418-resistant colonies were observed six weeks after transfection.

#### Measurement of Mediator Release

The release of 5-HT from mast cells was measured as previously described (Coleman et al., *J. Immunol.* 150:556-562 (1993)). Specifically,  $^3\text{H}$ -hydroxytryptamine creatinine sulfate (25.2 Ci/ mmol) (NEN) was added to mast cells 2 hours before stimulation. The cells were washed to remove unincorporated  $^3\text{H}$ -5-HT and stimulated with IgE and specific antigen as described above. Release of radioactivity in supernatant fractions (150  $\mu\text{l}$ ) was determined after stimulation, and net percentage release was calculated as  $[(a-b)/c] \times 100$ , where "a" is radioactivity released by stimulated cells, "b" is radioactivity released by unstimulated cells, and "c" is

-52-

total incorporated cellular radioactivity that was measured after cells were lysed in 0.05% Triton X-100 (Sigma). IL-6 release was assayed using enzyme-linked immunosorbent assay ELISA kits (ENDOGEN, Boston, MA) according to the manufacturer's instructions. Release of  $\beta$ -hexosaminidase from transfecants was measured on an ELISA reader using *p*-nitrophenyl-N-acetyl- $\beta$ -D-glucosamine (Sigma) as the substrate as described by Berger in Immunology Methods Manual Vol. 3 (ed. 5 Lefkovits, I.):1436-1440 (Academic Press, San Diego, 10 1997).

#### Kinase Assays

The p44/p42 Erk-MAP kinase activity was assayed 15 using the MAP Kinase Assay Kit (New England Biolabs, Beverly, MA). Specifically, cells were lysed by sonication in 0.5 ml of cell lysis buffer (20 mM Tris 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerolphosphate, 1 20 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml leupeptin, 1 mM PMSF). 0.2 ml of cell lysate was then incubated with the phospho-specific p44/p42MAP kinase antibody (1:100 dilution) overnight at 4°C and subsequently with Protein A/G PLUS-Agarose 25 (Santa Cruz Biotechnology, Sant Cruz, CA) for 3 hours at 4°C. Immunoprecipitates were incubated in 50  $\mu$ l 1X kinase buffer (25 mM Tris 7.5, 5 mM  $\beta$ -glycerolphosphate, 2 mM DTT, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM MgCl<sub>2</sub>) supplemented with 30 100 mM ATP and 1  $\mu$ g of Elk1 fusion protein substrate for 30 minutes at 30°C. The reaction mixture was subjected 30 to 12% SDS-PAGE gel electrophoresis, and then electroblotted onto Immobilon-P transfer membrane

-53-

(Millipore, Bedford, MA). Membranes were incubated with the phospho-specific Elk1 (Ser383) antibody (1:1000 dilution), and the antibody was visualized with horseradish peroxidase-conjugated anti-rabbit IgG 5 secondary antibody using the Phototope-HRP Western Detection Kit (New England Biolabs, Beverly, MA). The films were subsequently scanned using a densitometer (Molecular Dynamics, Sunnyvale, CA) and specific signals for phospho-Elk1 were quantitated by ImageQuant software 10 (Molecular Dynamics).

The activation of JNK activity was assayed using the SAPK/JNK Assay Kit (New England BioLabs). Specifically, total JNK was immunoprecipitated using an N-terminal c-Jun (1-89) fusion protein bound to 15 glutathione sepharose beads and incubated with ATP in the presence of kinase buffer. The phosphorylation of c-Jun by JNK was measured by Western blotting using a phospho-specific c-Jun antibody.

The activation of p38 MAP kinase activity was 20 assayed using the p38 MAP Kinase Assay Kit (New England BioLabs). Specifically, total p38 MAP kinase was immunoprecipitated using a p38 MAP kinase antibody and incubated with GST-ATF-2 fusion protein in the presence of ATP and kinase buffer. Phosphorylation of ATF-2 was 25 measured by Western blotting using a phospho-specific ATF-2 antibody and ECL.

#### **Statistical Analysis**

The results for differences in mediator release 30 were analyzed for statistical significance by the

-54-

unpaired Student's *t* test (two-tailed). All results are expressed as the arithmetic mean  $\pm$  SEM.

## RESULTS

### 5 Identification of a Differentially-Expressed mRNA in Mouse Mast Cells Stimulated with IgE and Antigen

To isolate genes involved in Fc $\epsilon$ RI-mediated signaling, the C1.MC/C57.1 cell line, a cloned growth factor-independent mouse mast cell line of BALB/c origin (Young et al., *Proc. Natl. Acad. Sci. USA* 84:9175-9179 (1987); Tsai et al., *FASEB J.* 10, Abstract A1268 (1996)), was used. It has been reported that C1.MC/C57.1 cells can exhibit many functional cellular responses characteristic of activated mast cells, including the release of pre-formed mediators and cytokines upon Fc $\epsilon$ RI receptor crosslinking (Gordon and Galli, *J. Exp. Med.* 174:103-107 (1991)). C1.MC/C57.1 mast cells were sensitized with anti-DNP IgE mAb and challenged with DNP<sub>30-40</sub>HSA or vehicle alone for 30 minutes, 1 hour, or 2 hours. Total RNAs were extracted from the stimulated or unstimulated mast cells, and subjected to mRNA differential display analysis as described (Liang and Pardee, *Science* 257:967-971 (1992); Liang et al., *Nucl. Acids Res.* 21:3269-3275 (1993)). Twenty-six differential display experiments were performed using different combinations of 3' anchored primers and 5' arbitrary primers and different RNA isolates from various time intervals of stimulation. Total RNA was extracted from C1.MC/C57 mast cells that had been activated by IgE and specific antigen for 1 hour, and then amplified using T<sub>12</sub>MT anchored primers and

-55-

the AP-5 arbitrary primer (5'-GTTGCGATCC) (SEQ ID NO: 7). Several differentially expressed bands were consistently detected, including band 60-4.

Band 60-4 was excised from the gel, reamplified, 5 and used as probe for subsequent Northern blot analysis. The 60-4 cDNA probe hybridized to a 2.8 kb mRNA whose expression was rapidly increased in C1.MC/C57.1 mouse mast cells that had been activated through the Fc $\epsilon$ RI for 30 minutes or 1 hour, but returned to baseline levels 10 after 2 hours. The 60-4 probe was subsequently subcloned, and sequence analysis revealed a 328 base pair cDNA with no significant homology to any other entry in the Genbank database.

15 **Cloning and Structure of Murine Rin2**

The 60-4 probe was used to screen a BALB/c mouse bone marrow-derived cultured mast cell (BMCMC) cDNA library constructed in the Uni-ZAP XR vector (Stratagene). One positive clone (SY-6) with a 1.1 kb 20 insert was isolated. Sequencing of the entire SY-6 partial cDNA clone revealed no significant homology with any known genes. To facilitate the cloning of the full-length cDNA, the SY-6 clone was used as a probe to assess the expression pattern of the identified novel 25 gene transcript by Northern blot analysis. The expression of this novel mRNA appeared widespread, as it was detected in many tissues, including the heart, liver, kidney, lung, skeletal muscle, testis spleen and brain. Because the identified gene transcript was 30 expressed fairly abundantly in the brain, the SY-6 partial cDNA clone was used to screen a mouse brain cDNA

-56-

library (Stratagene) in an attempt to obtain the full-length cDNA clone. Three positive clones (SY-A, SY-B, SY-C) were identified after the tertiary screening, and clone SY-A appeared to contain an insert 5 (2.6 kb) of the appropriate predicted size (2.8 kb).

Sequencing of the entire SY-A clone (2664 base pairs) revealed a potential translational initiation codon (ATG) at base 60 and a stop codon (TGA) at base 1534. The predicted open reading frame (ORF) of 1476 10 base pairs encodes a predicted protein of 491 amino acids with a molecular mass of 56.9 kDa (Figure 3). To determine whether the SY-A cDNA could indeed express a protein of the predicted size, the SY-A clone was subcloned into a pGEM expression vector and subjected to 15 coupled *in vitro* transcription/translation in a wheat germ lysate system. A specific product of an apparent molecular mass of 60 kDa was detected. These results are thus consistent with the size of the predicted protein deduced from the predicted ORF. Moreover, 20 hydrophilicity plot (Kyte-Doolittle) analysis of the predicted ORF revealed a highly hydrophilic protein with a few short hydrophobic regions. Thus, the protein product of the predicted ORF is likely to be a soluble cytosolic protein.

25 The predicted amino acid sequence of SY-A cDNA was used to search available protein databases using the BLAST program network server. Over a particular stretch of amino acid sequence, the SY-A gene product was found to share significant sequence homology with members of a 30 putative GTPase-binding protein family such as Vps9p (BLAST P= 2.2e-19), JC265 (P= 5.2e-05), and Rin1 (P= 4.1e-05) (Figure 4). Vps9p is a recently characterized

-57-

yeast protein required for vacuolar protein sorting (Burd et al., *Mol. Cell. Biol.* 16:2369-2377 (1996)). The two mammalian proteins encoded by two closely related human cDNAs, JC265 and Rin1, have been 5 identified as inhibitors of an activated Ras2 allele in *S. cerevisiae* (Colicelli et al., *Proc. Natl. Acad. Sci. USA* 88:2913-2917 (1991); Han and Colicelli, *Mol. Cell. Biol.* 15:1318-1323 (1995)). Alignment of the amino acid sequences of these proteins with that of SY-A showed 10 that the highest homology was found in the GTPase binding homology (GBH) domain regions in which three GBH motifs have been described (Burd et al., *Mol. Cell. Biol.* 16:2369-2377 (1996)). Specifically, the SY-A protein contains a 148-amino acid sequence which is 34% 15 identical (51/148) and 22% similar (33/148) to the respective GBH domains of Vps9p containing the three GBH motifs (Figure 4). Moreover, the SY-A gene product contains an amino acid sequence (SADDFLPTL; SEQ ID NO: 8) that matches well with the proposed GBH motif II 20 (GADXFLPVL; SEQ ID NO: 9), and another downstream sequence (GEDGYYFTN; SEQ ID NO: 10) that also aligns well with the proposed GBH motif III (GEXXXYLTS; SEQ ID NO: 11) (Burd et al., *Mol. Cell. Biol.* 16:2369-2377 (1996)) (Figure 4). Interestingly, the GBH motif II is 25 situated in a region of JC265 and Rin1 that has been previously suggested to be distantly related to several Ras-interacting proteins such as the human GAP, the yeast IRA1 and IRA2 proteins, NF1, and sar1 (Colicelli et al., *Proc. Natl. Acad. Sci. USA* 88:2913-2917 (1991)). 30 Because of its significant homology to the mammalian protein Rin1, the protein encoded by the SY-A cDNA is

herein called Rin2 (also called Rabex-5), and the gene encoding the protein is herein called *rin2*.

#### Interaction of Rin2 with Ras

5 Since Rin1 can suppress an activated Ras allele in yeast and interact directly with yeast Ras2p and with wild-type and activated human H-Ras (Han and Colicelli, *Mol. Cell. Biol.* 15:1318-1323 (1995); Han, *Proc. Natl. Acad. Sci. USA* 94:4954-4959 (1997)), it was of interest  
10 to determine whether Rin2 could also interact directly with Ras. Using the yeast two-hybrid system with the entire open reading frame (residues 1-491) of murine Rin2 expressed as a GAL4-activation domain fusion protein and the wild-type murine H-Ras expressed as the  
15 DNA-binding fusion protein, it was demonstrated that murine Rin2 was able to interact with wild-type murine H-Ras (Figure 12). Positive interactions with murine H-Ras could also be detected for the N-terminal and the C-terminal regions of Rin2, and for the region of Rin2  
20 containing the three GBH domains (residues 203-380). But the strength of interaction with murine H-Ras protein, as measured by a quantitative  $\beta$ -galactosidase assay, appeared to be the strongest for the full-length murine Rin2 protein (Figure 12). However, unlike Rin1,  
25 full length murine Rin2 proteins did not appear to bind significantly to the wild-type yeast Ras2p, nor did any of the three truncated fusion protein constructs representing the three different regions of the Rin2 protein. These data indicate that Rin2 can interact  
30 with Ras in ways that are distinct from those of Rin1.

-59-

**Decreased Expression of Rin2 Enhances Signaling Responses Induced by Fc $\epsilon$ RI Aggregation in Mast Cells**

- The Fc $\epsilon$ RI-induced activation of Ras in mast cells results in an increase in transcriptional activity of 5 the transcription factor Elk-1 (Turner and Cantrell, *J. Exp. Med.* 185:43-53 (1997)), which is a regulator of the serum response element in the promoter region of immediate early response genes such as c-fos (Treisman, *Curr. Opin. Genet. Dev.* 4:96-101 (1994)). The 10 transcription activation of Elk-1 is mediated by the Ras/Raf-1/MEK/Erk-MAP kinases pathway (Treisman, *Curr. Opin. Cell Biol.* 8:205-215 (1996)). As described herein, rin2 mRNA was identified by virtue of its enhanced expression in mast cells that are activated by 15 Fc $\epsilon$ RI aggregation. Because Rin1 has been indicated as a negative effector of Ras, the ability of Rin2 to suppress the signaling responses initiated by Ras in mouse mast cells activated by Fc $\epsilon$ RI ligation was assessed.
- 20 The SY-A cDNA was inserted into a CMV expression vector (pBK-CMV) in the antisense orientation, and the resulting plasmid (pBK-CMV-SYA-AS) was stably transfected into mouse C1.MC/C57.1 mast cells. From two independent transfections, eight separate stable lines 25 of antisense Rin2 transfectants and seven separate stable lines of control CMV vector transfectants were established.
- The G418-resistant colonies obtained were stimulated with IgE and antigen, and the pattern of 30 expression of early response genes such as c-fos was assessed using Northern blot analysis. Transfection of

-60-

antisense *rin2* significantly enhanced the expression of mRNA of *c-fos* in mouse mast cells activated by Fc $\epsilon$ RI crosslinking. In mouse mast cells transfected with the control CMV plasmid, *c-fos* mRNA expression was rapidly induced by 15 or 30 minutes after IgE and antigen-dependent stimulation, but returned to undetectable or basal levels by 1 hour after stimulation. In contrast, in mouse mast cells transfected with the *rin2* antisense construct, not only was a significantly higher level of *c-fos* expression observed at 15 or 30 minutes after IgE and antigen-dependent stimulation, but a significant and sustained induction of *c-fos* message was still detectable in these activated mast cells at 1 hour after stimulation.

Similarly, the activation of Erk-MAP kinase activity induced by Fc $\epsilon$ RI activation was also greatly potentiated in C1.MC/C57.1 mast cells that had been stably transfected with the antisense *rin2* construct (Figure 5). Consistent with results reported previously (Tsai et al., *Eur. J. Immunol.* 23:3286-3291 (1993)), Fc $\epsilon$ RI crosslinking caused a rapid activation of Erk-MAP kinase activity in mouse mast cells which had been transfected with the control plasmid 30 minutes after stimulation (2.2 fold increase). The activation persisted for 2 hours (approximately 1.5 fold increase) and then declined to basal levels by 3 hours after stimulation (1.0 fold). In contrast, in mouse mast cells transfected with the *rin2* antisense plasmid, a significantly higher level of Erk-MAP kinase activation was induced by Fc $\epsilon$ RI-dependent stimulation for 30 minutes (4.0 fold), 1 hour (3.6 fold), or 2 hours (2.2

-61-

fold) after the addition of antigen, and such Erk-MAP kinase activation was still sustained at 3 hours (1.3 fold).

Stimulation of mast cells by Fc $\epsilon$ RI aggregation  
5 leads to the activation of the Ras-mediated ERK-MAP kinase, JNK, and p38 signaling pathways (Tsai et al., *Eur. J. Immunol.* 23:3286-3291 (1993); Hirasawa et al., *J. Biol. Chem.* 270:10960-10967 (1995); Zhang et al., *J. Biol. Chem.* 272:13397-13402 (1997); Ishizuka et al.,  
10 *Proc. Natl. Acad. Sci. USA* 94:6358-6363 (1997); Kawakami et al., *J. Immunol.* 161:1795-1802 (1998)), and activation of the ERK-MAP kinases and JNK in turn regulate the synthesis and release of cytokines from these cells. Fc $\epsilon$ RI crosslinking caused a rapid  
15 activation of ERK-MAP kinase activity in control transfected C1.MC/C57.1 cells by 30 minutes to 2 hours after stimulation, which declined to basal levels by 3 hours after stimulation. In contrast, in the Rin2 antisense transfected mast cells, a significantly higher  
20 level of ERK-MAP kinase activation was induced by Fc $\epsilon$ RI stimulation 30 minutes to 2 hours after antigen challenge, and high levels of ERK-MAP kinase activation were still sustained at 3 hours. Antisense Rin2 expression also significantly potentiated the levels of  
25 activation of JNK (Figure 14) and p38 MAP kinase (Figure 15) induced by Fc $\epsilon$ RI aggregation at 30 minutes to 3 hours after stimulation. Taken together, the results of these studies indicate that reduced expression of Rin2 can result in marked potentiation of the Ras-mediated  
30 signaling responses in mast cells, and suggest that Rin2 functions as a Ras inhibitor/effectector.

-62-

**Decreased Expression of Rin2 Potentiates Release of Preformed Mediator from Mast Cells Activated by Fc $\epsilon$ RI Aggregation**

Since reduced expression of Rin2 can potentiate some of the intracellular signaling responses initiated by Fc $\epsilon$ RI-dependent mast cell activation, the effect of decreased Rin2 expression on the cells' secretory responses to Fc $\epsilon$ RI-dependent activation was also assessed. C1.MC/C57.1 mast cells which had been transfected with the *rin2* antisense expression or control vectors were stimulated with IgE and DNP<sub>30-40</sub>HSA. As shown in Figure 6, mouse mast cells stably transfected with the *rin2* antisense construct released significantly higher levels of the preformed mediator, 5-HT, than did mast cells transfected with the pBK-CMV vector only. This effect was observed at all concentrations of DNP antigen tested (1, 10, 50, or 100 ng/ml DNP<sub>30-40</sub>HSA), indicating that the potentiating effect of *rin2* antisense expression was not antigen dose-dependent.

**Decreased Expression of Rin2 Potentiates Cytokine Release from Mast Cells Activated by Fc $\epsilon$ RI Aggregation**

Crosslinking of Fc $\epsilon$ RI receptors not only induces mast cells to release preformed mediators such as 5-HT, but also induces these cells to secrete cytokines (Gordon et al., *Immunol. Today* 11:458-464 (1990); Paul et al., *Adv. Immunol.* 53:1-29 (1993)). Thus, the effect of changes in Rin2 expression on the Fc $\epsilon$ RI-dependent release of IL-6 from mouse mast cells was also assessed. As illustrated in Figure 7A, the release of IL-6 from

-63-

mast cells 2, 4, or 6 hours after Fc $\epsilon$ RI aggregation (50 ng/ml) was enhanced dramatically by the transfection of the cells with the *rin2* antisense expression vector, as compared with responses elicited in mast cells  
5 transfected with the control CMV vector. This potentiating effect was more pronounced (greater than 20-fold increase) in cells which had been stimulated for longer time intervals (4-6 hours). Furthermore, when the transfected cells were stimulated by different  
10 concentrations of DNP<sub>30-40</sub>HSA for 6 hours, mouse mast cells which had been transfected with the *rin2* antisense construct released significantly higher levels of IL-6 than did mast cells which had been transfected with the control vector at each concentration of antigen tested  
15 (Figure 7B).

As illustrated in Figure 13A, there were no significant differences between the amounts of  $\beta$ -hexosaminidase that were released from the antisense or control transfectants in response to Fc $\epsilon$ RI aggregation.  
20 This result is consistent with findings that the release of pre-formed mediators can occur independently of signaling pathways involving Ras/MAP kinase (Hirasawa et al., *J. Biol. Chem.* 270:10960-10967 (1995); Zhang et al., *J. Biol. Chem.* 272:13397-13402 (1997)). On the  
25 other hand, release of the cytokine IL-6 from the mast cell transfectants 6 hours after Fc $\epsilon$ RI aggregation was significantly enhanced (by a mean value of approximately 70%) in the antisense group (Figure 13A). The time course of this effect is shown in Figure 13B. In  
30 general, those antisense transfectants which exhibited the largest and most sustained reduction in Rin2 protein

-64-

expression after Fc $\epsilon$ RI aggregation also exhibited the greatest enhancement of IL-6 production (Figure 13C).

**Rin2 mRNA Expression is Up-regulated in Mouse Mast Cells**

5 Stimulated with Stem Cell Factor and in PC12 Cells

Stimulated with Nerve Growth Factor

To assess whether the enhanced expression of the *rin2* transcript was specific for the Fc $\epsilon$ RI-dependent signaling pathway in mast cells, Northern analysis was 10 performed to examine the expression of *rin2* mRNA in mouse bone marrow derived cultured mast cells (BMCMC) which had been stimulated with the growth factor, stem cell factor (SCF), the ligand for the c-kit receptor tyrosine kinase (Galli et al., *Adv. Immunol.* 55:1-96 15 (1994)). The widespread tissue distribution of *rin2* mRNA suggests that Rin2 may function as a signaling element in the functional responses of many cell types besides mast cells. Therefore, *rin2* expression was also analyzed in PC12 cells, an adrenal pheochromocytoma cell 20 line, which had been stimulated with nerve growth factor (NGF) (Greene and Tischler, *Proc. Natl. Acad. Sci. USA* 73:2424-2428 (1976)).

*rin2* mRNA expression was rapidly increased by 30 minutes to 1 hour after stimulation of BMCMCs with SCF 25 (50 ng/ml), but returned nearly to baseline levels after 2 hours. Expression of the *rin2* transcript was also significantly induced 30 minutes after NGF stimulation (50 ng/ml) in PC12 cells, and the enhanced expression of the transcript was still detectable 2 hours after 30 stimulation. Thus, SCF induced a transient enhancement of *rin2* mRNA expression in mast cells, but NGF elicited

-65-

a much more sustained activation of *rin2* expression in PC12 adrenal pheochromocytoma cells.

**Decreased Expression of Rin2 Enhances Cell Proliferation  
5 in PC12 Cells**

PC12 cells stimulated with NGF undergo neuronal differentiation, which is characterized by the extension of neurites and the cessation of cell proliferation (Greene and Tischler, *Proc. Natl. Acad. Sci. USA* 10 73:2424-2428 (1976)). Since NGF promoted a sustained induction of *rin2* mRNA expression in PC12 cells, the effects of reduced *rin2* expression on cell proliferation rate and NGF-induced neurite outgrowth were examined in these cells. The antisense expression vector pBK-CMV-15 SYA-AS was stably transfected into PC12 cells, and the transfected cells were plated onto collagen-coated plates and stimulated with NGF at 25, 50 or 100 ng/ml. After 3-5 days, neurite outgrowth was observed both in PC12 cells that had been transfected with the antisense 20 expression vector and in those cells that had been transfected with the control CMV vector. No detectable differences in the time of onset or the number of neurite-bearing cells could be observed between these populations of transfected cells. These results thus 25 indicate that Rin2 may not be directly involved with NGF-induced neuronal differentiation in PC12 cells. On the other hand, PC12 cells which had been transfected with the antisense expression vector and then maintained in their normal culture medium exhibited a significantly 30 higher rate of cell proliferation, as measured by a <sup>3</sup>H-thymidine assay, than did the cells which had been

-66-

transfected with the control pBK-CMV vector (Figure 8A). This effect was also observed when the transfected PC12 cells were stimulated with NGF (100 ng/ml) for 18 hours in medium containing 0.5% FCS (Figure 8B). Since NGF is known to be anti-mitogenic to PC12 cells (Greene and Tischler, *Proc. Natl. Acad. Sci. USA* 73:2424-2428 (1976)), these results are consistent with the hypothesis that Rin2 expression results in the inhibition of cell proliferation in PC12 cells and perhaps other neuronal cells.

**Rin2 mRNA Expression is Up-Regulated in EL-4 T Cells Stimulated via the T Cell Receptor Complex**

The binding of peptide antigen and major histocompatibility complex molecules to the T cell receptor (TCR) induces T cell activation, which includes the secretion of effector cytokines by the T cells and the induction of mitotic activity in the T cells themselves. Extensive evidence has shown that the activation of Ras is involved in some of the intracellular signaling pathways mediating T cell activation (Lowy and Willumsen, *Ann. Rev. Biochem.* 62:851-891 (1993)). To assess whether T cell activation could result in enhanced expression of the *rin2* transcript, Northern analysis was performed to examine the expression of *rin2* mRNA in EL-4 mouse T cells that had been stimulated via the TCR by incubating the cells with both anti-CD3 antibody (1 µg/ml) and anti-CD28 antibody (2 µg/ml). *rin2* mRNA expression was rapidly increased by 30 minutes to 1 hour after TCR stimulation, but declined nearly to baseline levels after 2 hours.

-67-

The time course of this effect on *rin2* mRNA expression in T cells is thus similar to that observed in mouse mast cells stimulated with SCF or IgE and antigen.

##### 5 EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such 10 equivalents are intended to be encompassed by the following claims:

-68-

CLAIMS

We claim:

1. An isolated Rin2 polypeptide which down-regulates functional responses elicited by Ras-dependent signaling pathways, or a derivative or portion thereof having Rin2 activity.
- 5 2. An isolated Rin2 polypeptide according to Claim 1, wherein the Ras-dependent signaling pathway is Fc $\epsilon$ RI aggregation.
- 10 3. An isolated Rin2 polypeptide according to Claim 1, wherein said Rin2 polypeptide comprises the amino acid sequence of SEQ ID NO: 2.
- 15 4. An isolated Rin2 polypeptide according to Claim 1, wherein said Rin2 polypeptide has the same amino acid sequence as endogenous Rin2.
- 20 5. An isolated Rin2 polypeptide according to Claim 1, wherein said Rin2 polypeptide comprises an amino acid sequence which is at least about 40% similar to the amino acid sequence of SEQ ID NO: 2.
- 25 6. An isolated nucleic acid molecule which encodes a Rin2 polypeptide which down-regulates functional responses elicited by Ras-dependent signaling pathways, or a derivative or portion thereof having Rin2 activity.
- 30

-69-

7. An isolated nucleic acid molecule according to  
Claim 6, wherein said nucleic acid molecule  
comprises the nucleotide sequence of SEQ ID NO: 1  
or the complement of SEQ ID NO: 1, or a derivative  
5 or portion thereof.
8. An isolated polypeptide encoded by a nucleic acid  
molecule according to Claim 7.
- 10 9. An isolated nucleic acid molecule according to  
Claim 6, wherein said nucleic acid molecule  
comprises one or more nucleotide sequences selected  
from the group consisting of:
  - a) SEQ ID NO: 12 or the complement of ID NO: 12;
  - 15 b) SEQ ID NO: 13 or the complement of SEQ ID  
NO: 13;
  - c) SEQ ID NO: 14 or the complement of SEQ ID NO:  
14;
  - d) SEQ ID NO: 15 or the complement of SEQ ID NO:  
20 15; and
  - e) derivatives or portions of (a)-(d).
10. An isolated protein encoded by an isolated nucleic  
acid molecule according to Claim 8.  
25
11. An isolated nucleic acid molecule according to  
Claim 6 comprising a nucleotide sequence which  
selectively hybridizes to all or a portion of SEQ  
ID NO: 1.
- 30 12. An isolated nucleic acid molecule according to  
Claim 6 comprising a nucleotide sequence which

-70-

selectively hybridizes to all or a portion of one or more nucleotide sequences selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15.

5

13. An isolated nucleic acid molecule according to Claim 6 comprising a nucleotide sequence which is at least about 75% identical to SEQ ID NO: 1.

10 14. An isolated nucleic acid molecule according to Claim 13 comprising a nucleotide sequence which is at least about 90% identical to SEQ ID NO: 1.

15 15. An isolated nucleic acid molecule according to Claim 6 comprising a nucleotide sequence which is at least about 75% identical to one or more nucleotide sequences selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15.

20

16. An isolated nucleic acid molecule according to Claim 15 comprising a nucleotide sequence which is at least about 90% identical to one or more nucleotide sequences selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15.

25 17. A DNA construct comprising an isolated nucleic acid molecule according to Claim 6 operatively linked to 30 a regulatory sequence.

-71-

18. A recombinant host cell comprising an isolated nucleic acid molecule according to Claim 6 operatively linked to a regulatory sequence.
- 5 19. A method for preparing a Rin2 polypeptide, comprising culturing the recombinant host cell of Claim 18.
- 10 20. An antibody, or an antigen-binding fragment thereof, which selectively binds to the Rin2 polypeptide according to Claim 1, or a derivative or portion thereof having Rin2 activity.
- 15 21. A method for assaying the presence of a Rin2 polypeptide according to Claim 1 in a cell or cell lysate, comprising contacting said cell or cell lysate with an antibody which specifically binds to the Rin2 polypeptide.
- 20 22. A method of identifying an agent which alters activity of a Rin2 polypeptide according to Claim 1, comprising the steps of:
  - (a) contacting a cell containing the Rin2 polypeptide, or a derivative or portion thereof having Rin2 activity, with a stimulus which activates at least one Ras-dependent pathway in said cell, in the presence of the agent to be tested; and
  - (b) identifying alteration of Rin2 activity.
- 25 30 23. A method according to Claim 22, wherein the stimulus is selected from the group consisting of

-72-

nerve growth factor, stem cell factor, peptide antigen and major histocompatibility complex molecules, and IgE and specific antigen.

- 5 24. A novel agent which alters Rin2 activity identified according to the method of Claim 23.
25. A method of enhancing activity of a Rin2 polypeptide according to Claim 1, comprising  
10 contacting said Rin2 polypeptide, or a cell containing said Rin2 polypeptide, with an agent that enhances the activity of Rin2.
26. A method of inhibiting a functional response induced by a Ras-dependent signaling pathway in a  
15 cell, comprising contacting said cell with an agent which enhances the activity of a Rin2 polypeptide.
27. A method according to Claim 26, wherein the cell  
20 expresses a receptor having a signaling pathway regulated by Rin2.
28. A method according to Claim 27, wherein the receptor is selected from the group consisting of  
25 Fc $\epsilon$ RI, TrkA, c-kit and T cell receptor.
29. A method according to Claim 26, wherein the functional response is one or more responses selected from the group consisting of activation of  
30 ERK-MAP kinase, activation of JNK kinase and activation of p38 MAP kinase.

-73-

30. A method according to Claim 26, wherein the functional response is a cellular secretory response.
- 5 31. A method according to Claim 30, wherein the cellular secretory response is release of preformed mediators, lipid mediators and/or cytokines.
- 10 32. A method of inhibiting a Ras-dependent signaling pathway in a cell, comprising contacting said cell with an agent which enhances the activity of a Rin2 polypeptide.
- 15 33. A method of inhibiting cellular proliferation in a cell, comprising contacting said cell with an agent which enhances the activity of a Rin2 polypeptide.
- 20 34. A method according to Claim 33, wherein the cell expresses the TrkA receptor for nerve growth factor.
- 25 35. A method of inhibiting Ras-dependent signaling in a mammal comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.
- 30 36. A method of inhibiting a functional response induced by a Ras-dependent signaling pathway in a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

- 74 -

37. A method of inhibiting Ras-dependent signaling in the mast cells of a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

5

38. A method of inhibiting Ras-dependent signaling in T cells of a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

10

39. A method of inhibiting Ras-dependent cell proliferation in a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

15

40. A method of treating an allergic condition in a mammal comprising administering to the mammal an effective amount of an agent which enhances the activity of the Rin2 polypeptide.

20

41. A method according to Claim 40, wherein the allergic condition is asthma, hay fever or atopic eczema.

25

42. A method of treating a disorder selected from the group consisting of Ras-dependent cancer, Ras-dependent neoplastic cellular proliferation and Ras-dependent unwanted non-neoplastic cellular proliferation in a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of a Rin2 polypeptide.

30

-75-

43. A method of inhibiting functional responses induced by T cell receptor-dependent activation of T cells in a mammal comprising administering to the mammal an effective amount of an agent which enhances the  
5 activity of Rin2.
44. A method of treating a disorder selected from the group consisting of autoimmune disorder, T cell-associated disorder, T cell-dependent graft  
10 rejection and graft-versus-host disease in a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of a Rin2 polypeptide.
- 15 45. A method according to Claim 44, wherein the disorder is selected from the group consisting of autoimmune (Type I) diabetes mellitus, rheumatoid arthritis, ankylosing spondylitis, sarcoidosis, Sjögren's syndrome, multiple sclerosis,  
20 inflammatory bowel disease, Crohn's disease, ulcerative colitis, dermatomyositis, scleroderma, polymyositis, systemic lupus erythematosus, biliary cirrhosis, autoimmune thyroiditis, autoimmune hepatitis, psoriasis, contact sensitivity and  
25 atopic dermatitis.
46. A method of inhibiting activity of a Rin2 polypeptide according to Claim 1, comprising contacting said Rin2 polypeptide, or a cell  
30 containing said Rin2 polypeptide, with an agent that inhibits the activity of Rin2.

- 76 -

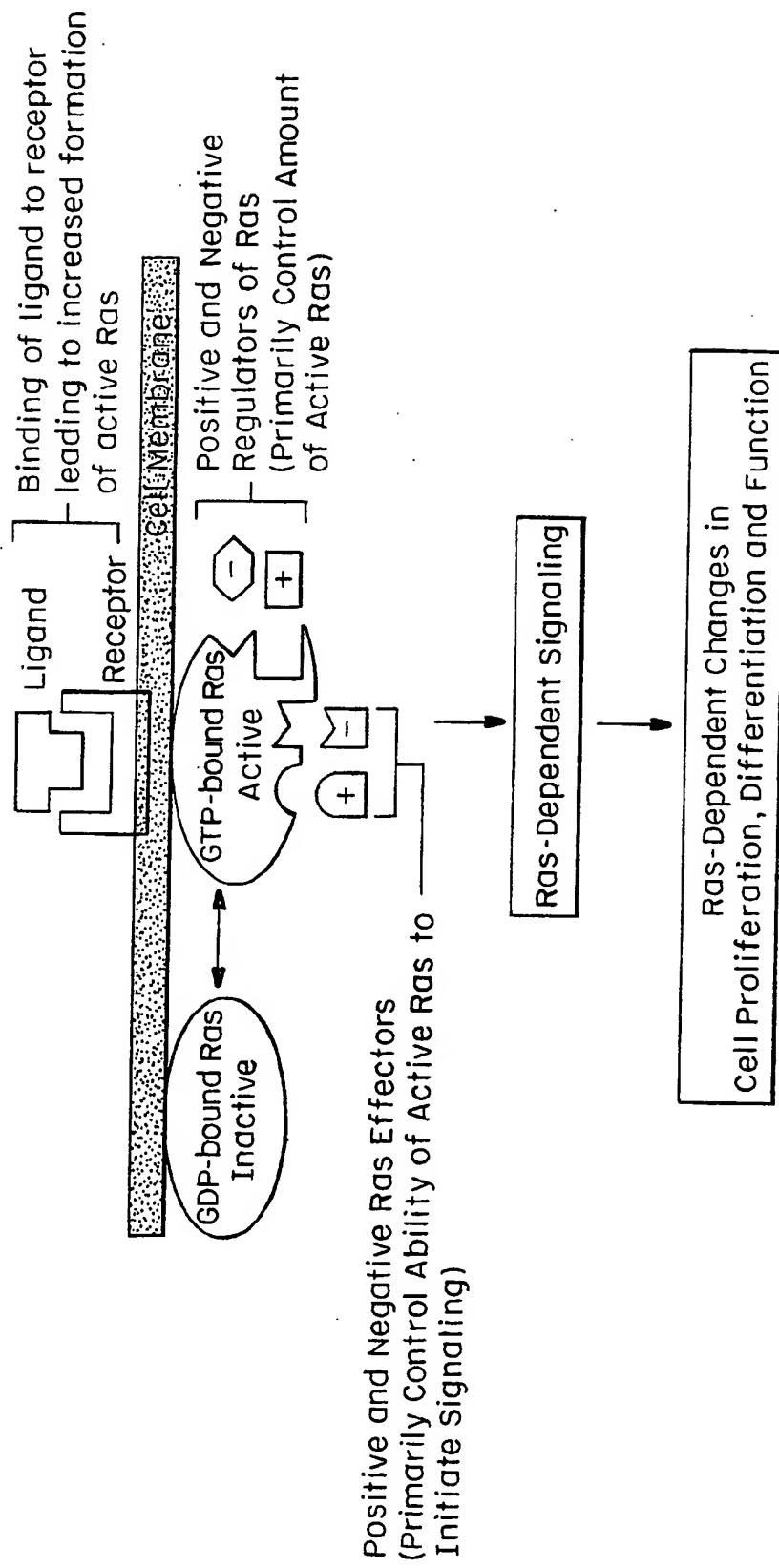
47. A method of enhancing a functional response induced by a Ras-dependent signaling pathway in a cell, comprising contacting said cell with an agent which inhibits the activity of a Rin2 polypeptide.
- 5           48. A method according to Claim 47, wherein the cell expresses a receptor having a signaling pathway regulated by Rin2.
- 10          49. A method according to Claim 48, wherein the receptor is selected from the group consisting of Fc $\epsilon$ RI, TrkA, c-kit and T cell receptor.
- 15          50. A method of enhancing a Ras-dependent signaling pathway in a cell, comprising contacting said cell with an agent which inhibits the activity of a Rin2 polypeptide.
- 20          51. A method of enhancing a functional response induced by a Ras-dependent signaling pathway in a mammal, comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.
- 25          52. A method of enhancing Ras-dependent signaling in a mammal comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.
- 30          53. A method of enhancing functional responses induced by T cell receptor-dependent activation of T cells in a mammal comprising administering to the mammal

-77-

an effective amount of an agent which inhibits the activity of Rin2.

54. A method of enhancing Ras-dependent signaling in  
5 the mast cells of a mammal, wherein the desired outcome is increased mast cell function, comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.
  
- 10 55. A method of enhancing Ras-dependent signaling in T cells of a mammal, wherein the desired outcome is increased T cell function, comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.
  
- 15 56. A method of enhancing Ras-dependent cell proliferation in a mammal, comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.
  
- 20 57. A method according to Claim 56, which produces enhanced wound healing, angiogenesis, and/or re-epithelialization.

25



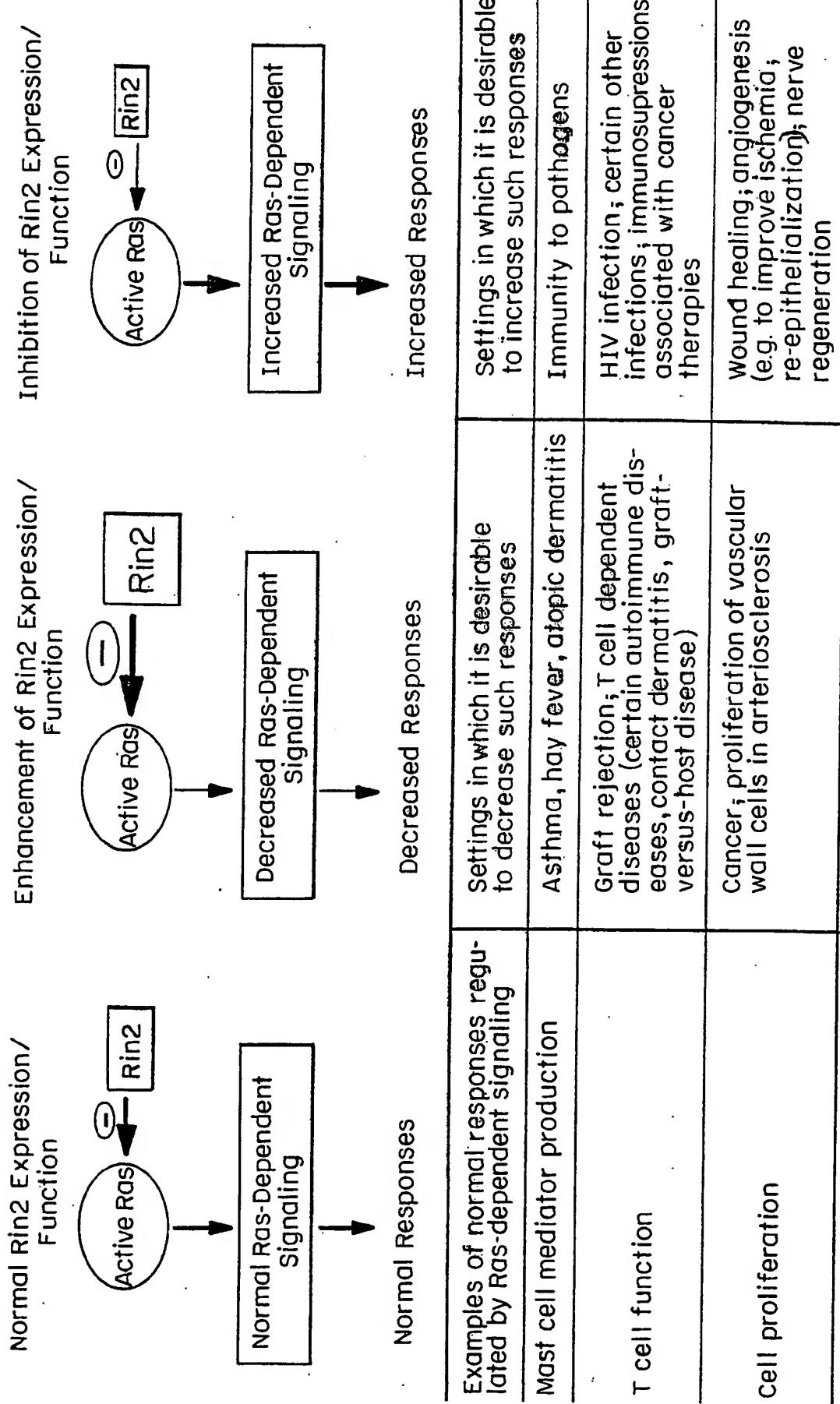


FIG. 2

**SUBSTITUTE SHEET (RULE 26)**

FIG. 3A

3B  
FIG

FIG. 4

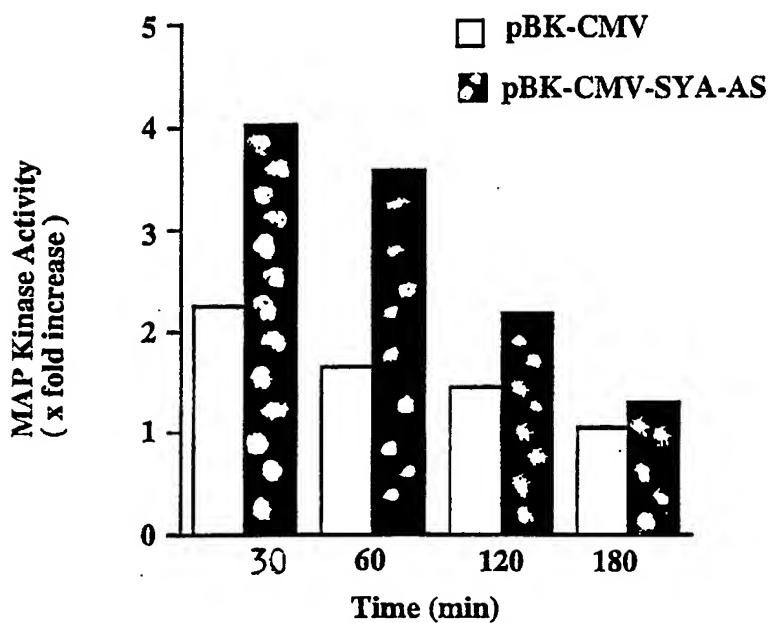


FIG. 5

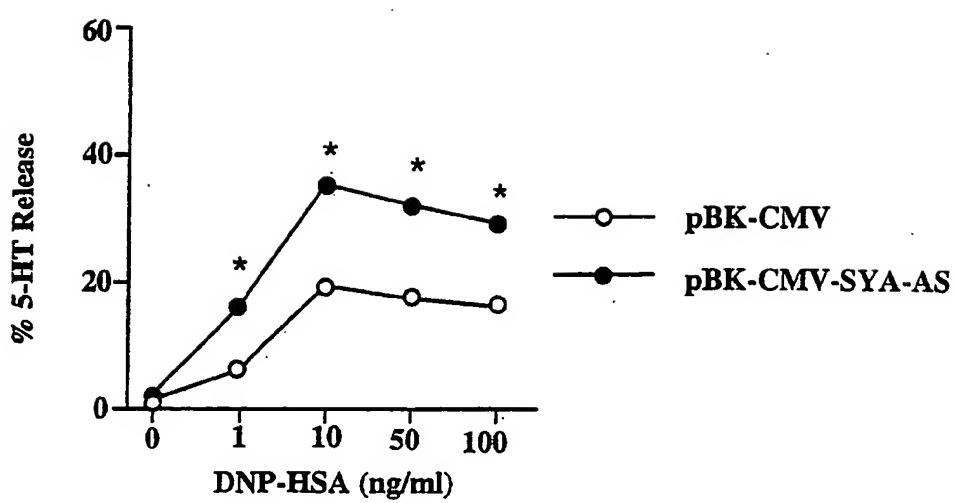


FIG. 6

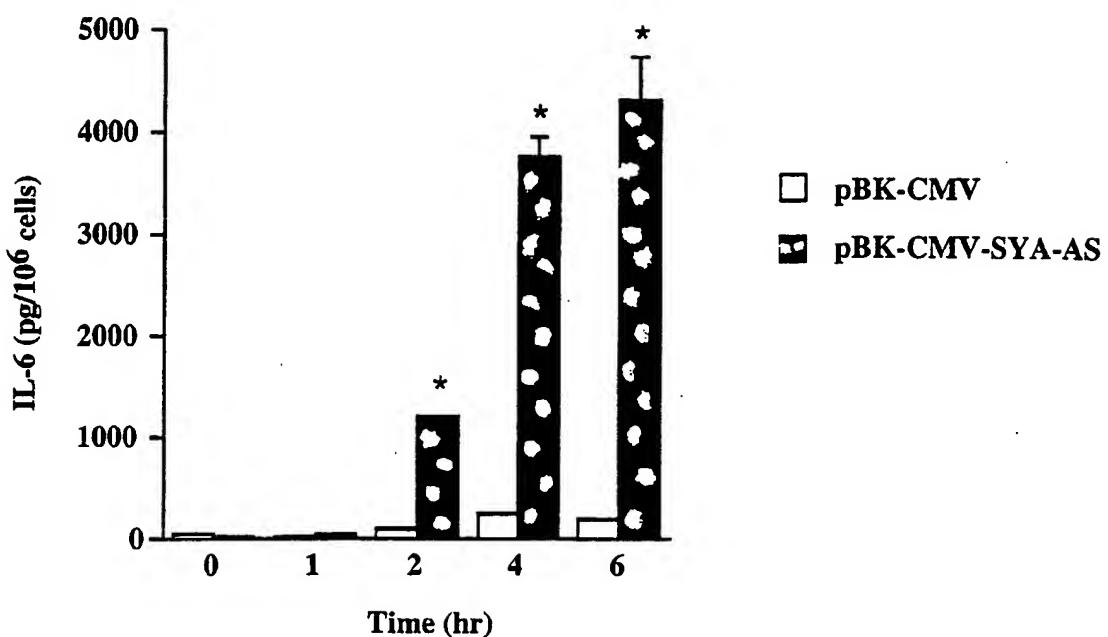


FIG. 7A

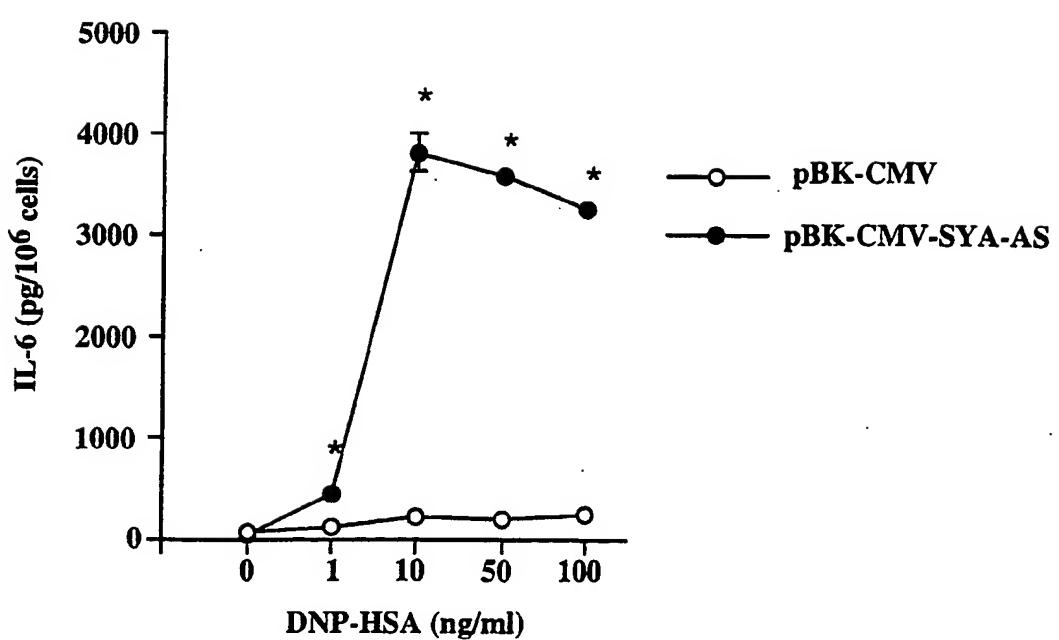
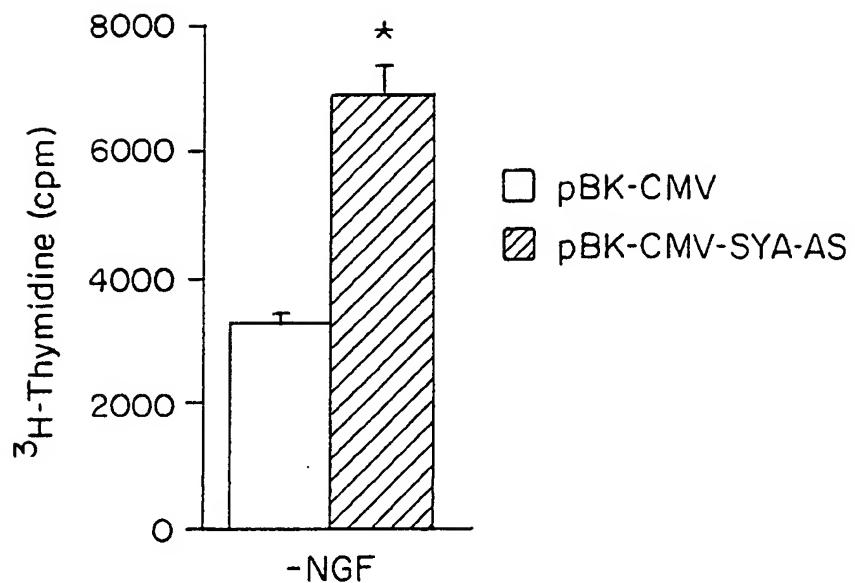
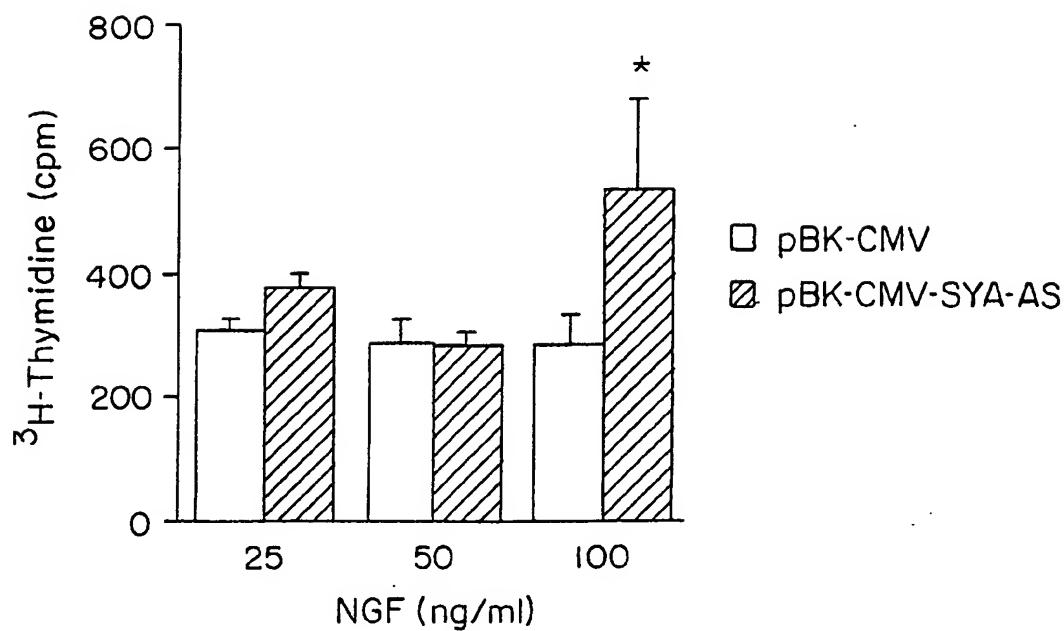
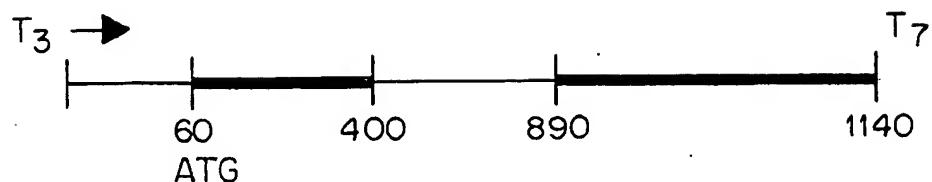


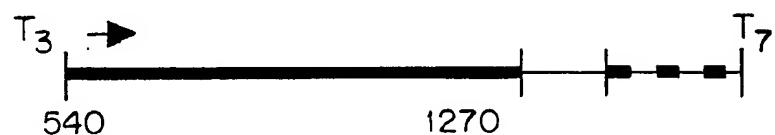
FIG. 7B

**FIG. 8A****FIG. 8B**

HRIN2-1B1=1.3kb



HRIN2-2B1=2.9kb



number=nt # in murine Rin2 sequence

■—■—■ 3' end sequenced

■ Coding region sequenced

**FIG. 9**

79.2% identity in 355 nt overlap; score: 883

Murine Rin2	60	70	80	90	100	110
-	AGGAAGAAGATGAGCCTGAAGTCCGACGGAGGGATTCAATGTGGATCAATCTGAGCTC					
-	: :					
-	AAGAAGAAGATGAGCCTTAGGTCTGAACGCCGAGGAATTCAATGTGGATC-----					
 HRIN2-1B1						
	120	130	140	150	160	170
-	CTGTGCAAGAAAGGATGCGGTTACTACGGCAACCCTGGCCTGGCAGGGTTCTGCTCCAAG					
-	: :					
-	CTGTGCAAGAAAGGATGTGGTTACTGTGGCAACCCTACCTGGCAGGGTTCTGCTCCAAG					
	180	190	200	210	220	230
-	TGCTGGAGGGAGGGAGTACCAACAAGGCCGGCAGAGGCAGATCCAAGAGGACTGGAACTG					
-	: :					
-	TGCTGGAGGGAAAGAGTAGCACAAAGCCAGGCAGAACGAGATTCAAGGAGTACTGGAGCTG					
	240	250	260	270	280	290
-	GCAGAACGACTTCAGCGGGAGGAGGAAGAGGGCTTCGGAGCAGCCAGAGCAGCCAGGA					
-	: :					
-	GTTAACGAACTCCNNCGGGAGGAAGAAGAG-CCTTTGCCANCAGTCAGAGCAGCCAGGG					
	300	310	320	330	340	350
-	GCCCAGTCCTCACCTTCTCAAGTTCTGGAGGAGAACGACCAATGAGAAAACCGAAAA					
-	: ..					
-	GCCCACATCCCTCATATTCTCCANCTTGAGGAAAGAAAACCAACNAGANCACCCNCNNG					
	360	370	380	390	400	
-	GTCACACACAGTGAAGAAGTTCTTCAGCGGCTCTTCCAGAGCTGGATCCAAGAAGG					
-	: :					
-	GTTACACACAGTGAANAAAT-CTTCAGTACGTCTTCCAGGGTCCGATCAAAAAAAAG					

FIG. 10A

78.6% identity in 266 nt overlap; score: 607

Murine Rin2 890 900 910 920 930 940  
 - ATTGAGATGGACT--CAAAGCGTGTGCCTCGGGACAAGCTGGCC-TGCATCACC-AGGTG  
 : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: :  
 HRIN2-1B1 ATTGAAATGGATTNCCAGGGGGTGTGCNTCGAGAAAAGCTGGCCNTGCNTCACCCAAGGG

950 960 970 980 990 1000  
 - CAGCAAGCACATCTTCATGCCATCAAGATCACCAAGAACGAGCCAGCCTCTGCCGATGA  
 : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: :  
 - CAGCAAGNANATTTTNGCCATCAAGATCACTTAGAACGAGCTGGCGTCAGCAGATGA

1010 1020 1030 1040 1050  
 - CTTCCCTGCC-ACCCCTGATCTACATCGCTCTGAAGGGCAACCCCCCTGCCCTGCAGTCCA  
 : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: :  
 - NTTTCTTCCCCACCCCTCATTTACATTGTTGAAGGGCAACCCCCAT-GCCTTCAGTTA

1060 1070 1080 1090 1100 1110  
 - ACATCCAGTACATCACTCGCTCTGCAACCCCAGCCCCCTCATGACGGCGAGGGATGGCT  
 : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: : :: :  
 - ATATCCAGTATATCACCGCGCTCTGCAATCCAAGCCACTGATGACTGGAGAGGGATGGCT

1120 1130 1140  
 - ACTACTTCACCAACCTGTG-CTGTGC  
 : :: : :: : :: : :: : :: :  
 - ACTATTTACCAATCTGAGGCTGGGC

FIG. 10B

83.5% identity in 746 nt overlap; score: 2027

FIG. 11A

## Murine Rin2/ Human HRIN2-2B1 clone (continued)

440	450	460	470	480	490
-----	-----	-----	-----	-----	-----

Murine Rin2	960	970	980	990	1000	1010
-	TCTTCATGCCATCAAGATCACCAAGAATGAGCCAGCCTCTGCCATGACTTCCTGCCA					
-	:::::::,::::::,::::::,::::::,::::::,::::::,::::::,::::::,::::::					
-	TCTTCATGCCATCAAGATCNCCANGAATGAGCCGGCTCTGCCATGANTTCCTCCCCA					
HRIN2-2B1						

1020	1030	1040	1050	1060	1070
-	CCCTGATCTACATCGCTCTGAAGGGCAACCCCCCTGCCGCAGTCCAACATCCAGTACA				
-	::::: :::::: : :: ::::::: :: ::::::: :: :: :: :: :: :: :: :: :				
-	CCCTCATCTACTT-GTTTGAAANGCA-CCCCCNC-CCTTCA-TCTAATATCCA-TATA				

1080	1090	1100	1110	1120	1130
-	TCACTCGCTCTGCAACCCA-GCCGGCTCATGACGGCGAGGATGGCTACTACTTCACC				
-	::::: :::::: : :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :				
-	TCACCGCGCTCTGCCATCCAAAGCCGACTGAT-ACTGGANAAGANGGCTACTA-TTC-CC				

1140	1150	1160	1170	1180	1190
-	AACCTGTGCTGTGCTGTGGCTTTCATTGAGAAAATTAGACGCCAGTCTTGAATTAAAGT				
-	:: ::::::: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :				
-	CATCTGTGCTGTGCGNTGGCTTTCATTGAAAAACTAAC-CCCNNTCTTGAATCTAATT				

1200	1210	1220	1230	1240	1250
-	CAGGAGGATTTGACCGGTACATGTCGGCCAGACATCCCCAGGAAGCAGGAGTCTGAG				
-	::::: :				
-	CNGGAAGAMTTGATCNCTACNTTTGGCCNGA-ACCCNCCNNGAAACCANAACNAA				

1260	1270
-	AGTTGGCCCCCGGAGGCCCTGCTTAGG
-	:: :
-	AATTGGTCCCCG-ANGCTTGCTTAGG
	790
	800

FIG. 11B

10            20            30            40

---

TNCCAANAAANATATTCAAGTNTTTGGGGGGCNAAATT 40  
 CACCCCNCGGTTAAAGGAACCAATTGATNTTTTAA 80  
 CAAAACNGGGAGTTTCCTNCGGGGGGGTAAATANG 120  
 GGGNAACCCAAAGATTNTGCNATTCAATGCACAGGNGGG 160  
 ATGTNAACTAAAAACGGAGTTAAATATTTAGGGGGTTG 200

210            220            230            240

---

ACAGCAACCTGCATNGTAGAACCTTTTTNTTCNGNG 240  
 GACNTNTATAACNTAAATATAACCATTGATGATTTNTTC 280  
 CATTCACTGACATCCACAGATTANGCAGCTATACTGTGA 320  
 AATCGTGATGAGGCCAGGGCACCGTTAGAACAAAC 360  
 GTCACTTCACACAGGCAGGTGAGAAAGGTTCTTGCTTT 400

410            420            430            440

---

TCCAGTATCTTCTTAAGGATGGAGCCCCAAAATTGCAGAGC 440  
 AGTAACCTTGGAAATAAAACCAGGGTGGGTATAAAACTTCT 480  
 TATTCTTAAATTACATATAAGATCTATTAAGCTTGACAC 520  
 ATCTGTGTCACTCACGCACTGAAGACAGGAAGCAGTTCACT 560  
 GAGTCAGCTGGTCTTCAAGCTCGCACAGAAGGTGATAAGT 600

610            620            630            640

---

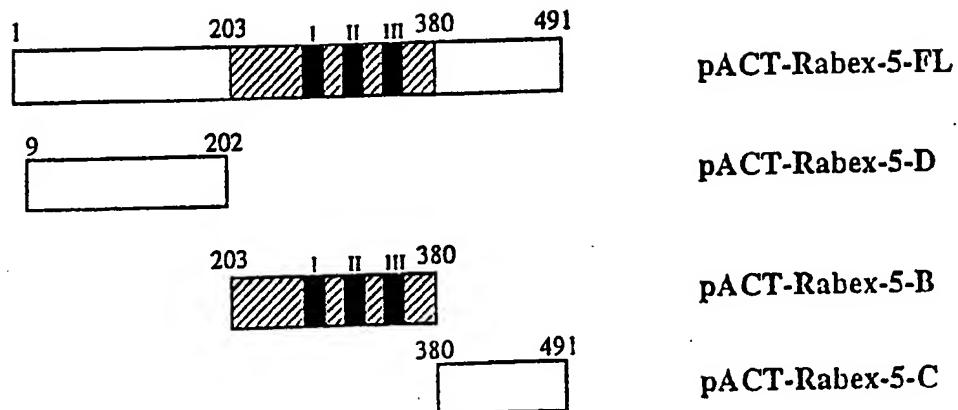
TACTATCAAATGCCAGTGAGAATCTTCTTATAGAATAACC 640  
 TGGGCCCAAGTGATTTAGTACAAAACCTGCCCTTCTTG 680  
 GTTTAATTTCTATGTGCTTTAGGTGTGAATCCAGATAT 720  
 GCAGTCTTAATTCTTGGAAATACACAGTTGTTAGTT 760  
 ACTGTACACTCTGTTGTTCAATAAACTGCATATCAACTT 800

810            820            830            840

---

CCCCCCCAAAAAAAAAAAAAAAA 822

FIG. 11C



DNA-binding hybrid	Activation domain hybrid	Colony color	Relative $\beta$ -Gal units
pAS2-mHRas	pACT-Rabex-5-FL	Blue	229
pAS2-mHRas	pACT-Rabex-5-D	Blue	14
pAS2-mHRas	pACT-Rabex-5-B	Blue	24
pAS2-mHRas	pACT-Rabex-5-C	Blue	138
pAS2-yRas2p	pACT-Rabex-5-FL	White	6
pAS2-yRas2p	pACT-Rabex-5-D	White	4
pAS2-yRas2p	pACT-Rabex-5-B	White	<1
pAS2-yRas2p	pACT-Rabex-5-C	White	<1

FIG. 12

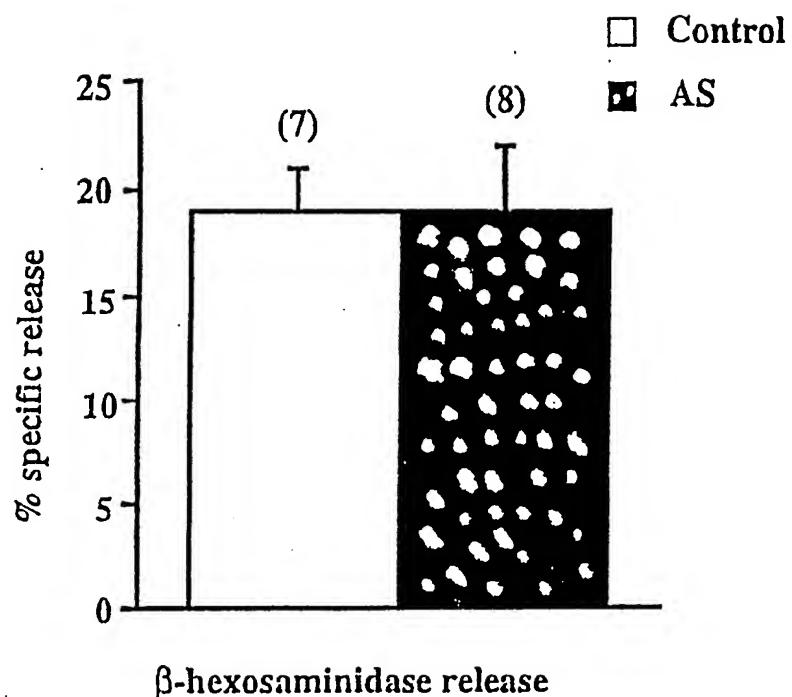


FIG. 13A

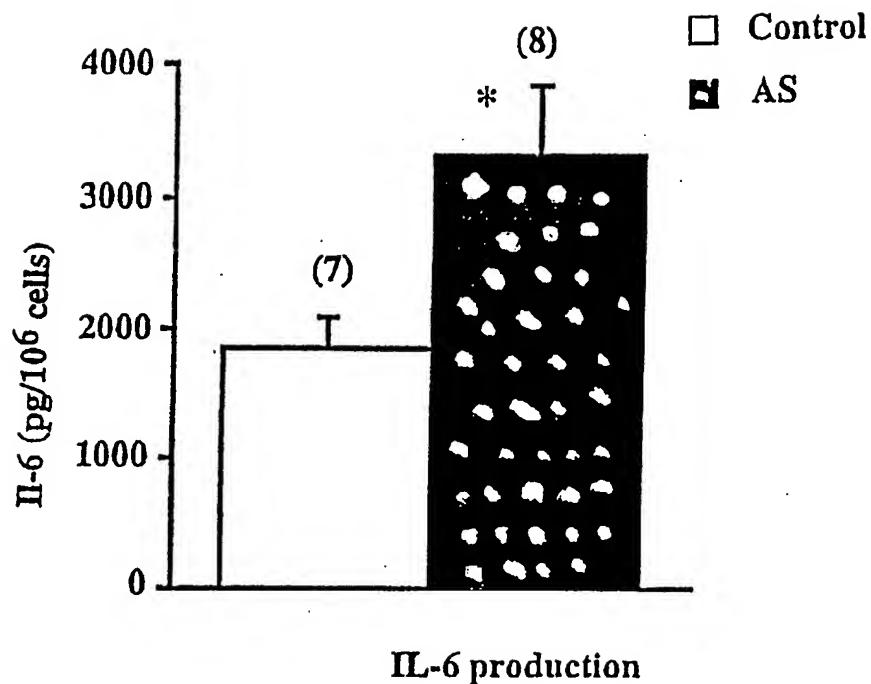


FIG. 13B

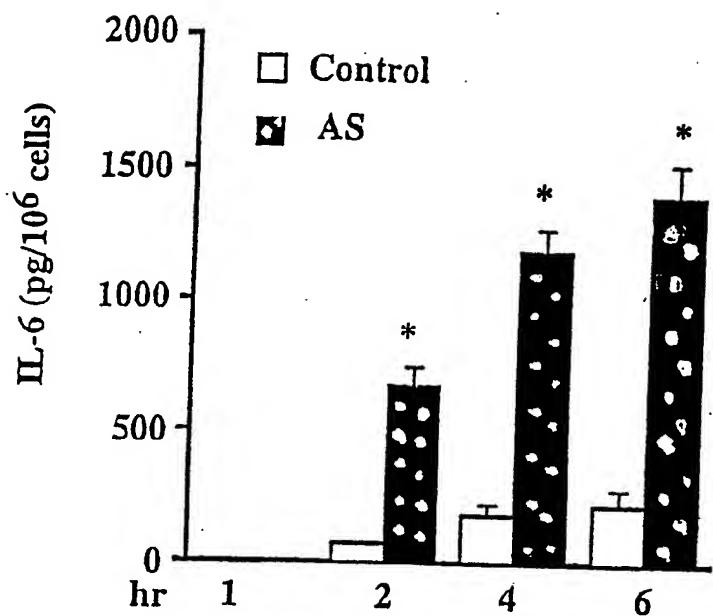


FIG. 13C

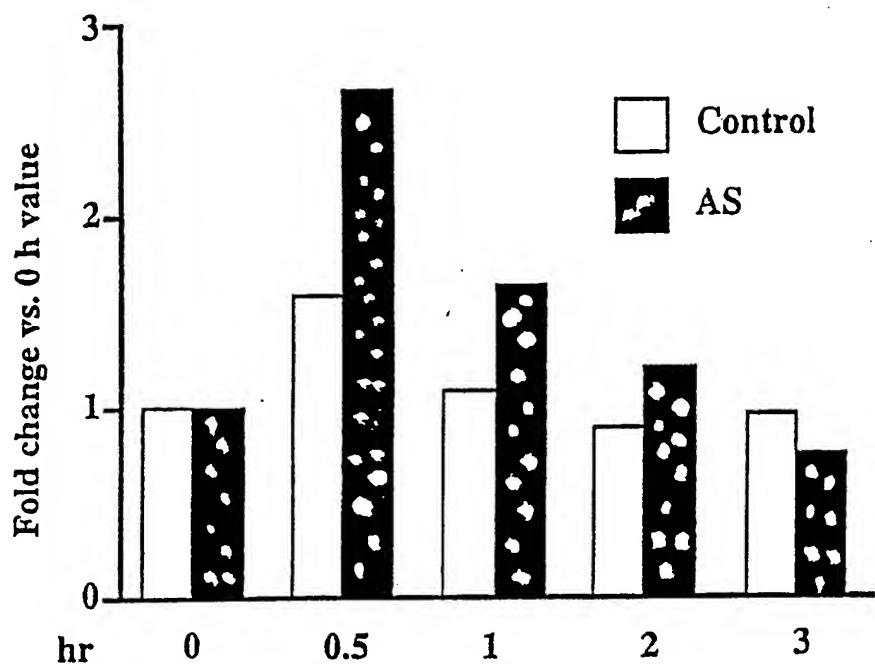


FIG. 14

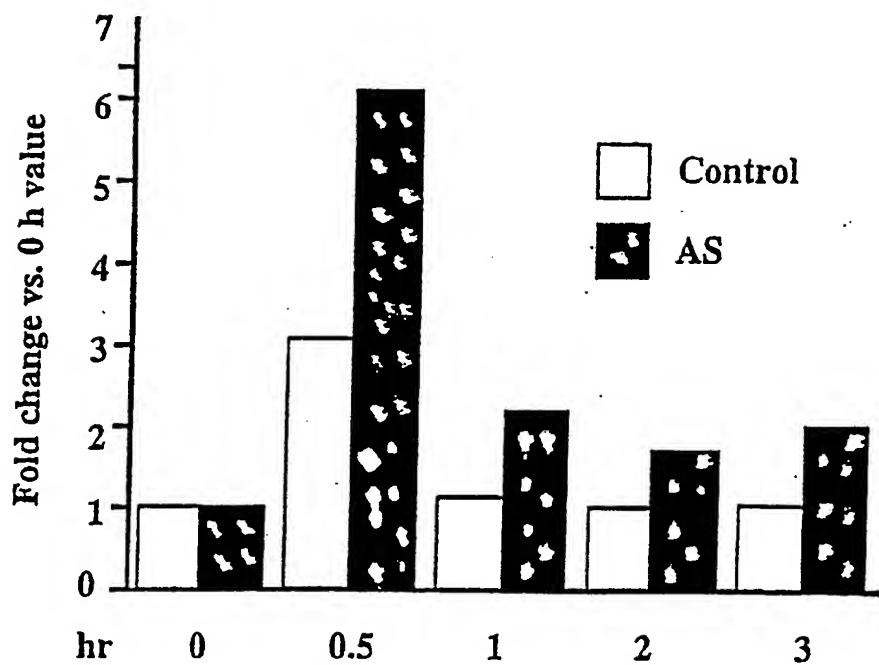


FIG. 15

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/47 C07K16/18 C12Q1/68 G01N33/50  
G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N C07K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 666 314 A (COLD SPRING HARBOR LAB) 9 August 1995 SEQ ID NOS: 13-16 see page 12, line 53 - line 56 ----	9,10,12, 15,16
X	J. COLICELLI ET AL.: "Expression of three mammalian cDNAs that interfere with RAS function in <i>Saccharomyces cerevisiae</i> " PROC. NATL. ACAD. SCI., vol. 88, April 1991, pages 2913-2917, XP002087363 NATL. ACAD. SCI., WASHINGTON, DC, US; see the whole document ----	9,10,12, 15,16

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

10 December 1998

28/12/1998

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Authorized officer

HORNIG, H

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L. HAN ET AL.: "Protein binding and signalling properties of RIN1 suggest a unique effector function" PROC. NATL. ACAD. SCI., vol. 94, May 1997, pages 4954-4959, XP002087364 NATL. ACAD. SCI., WASHINGTON, DC, US; cited in the application see the whole document ----	9,10,12, 15,16
X	C.G. BURD ET AL. : "A yeast protein related to a mammalian ras-binding protein, Vps9p, is required for localization of vacuolar proteins" MOL. CELL. BIOL., vol. 16, no. 5, May 1996, pages 2369-2377, XP002087365 ASM WASHINGTON, DC, US cited in the application see the whole document ----	9,10,12, 15,16
A	H. STENMARK ET AL.: "Rabaptin-5 is a direct effector of the small GTPase Rab5 in endocytic membrane fusion" CELL, vol. 83, 3 November 1995, pages 423-432, XP002087366 CELL PRESS, CAMBRIDGE, MA, US; see the whole document ----	1-23
P, X	HORIUCHI H ET AL: "A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function." CELL, (1997 SEP 19) 90 (6) 1149-59. JOURNAL CODE: C04. ISSN: 0092-8674., XP002087367 see the whole document -----	1-8,11, 13,14, 17-21

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/19056

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: 24-54 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
Please see Further Information sheet PCT/ISA/210
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Claims Nos.: 24-54

Claims 24 relating to agents which alter Rin2 activity relating to claims 25-57 could not be searched as the subject matters were not sufficiently disclosed.

Furthermore Claims 9,10,12,15,16, make no sense SEQ ID NOS.  
12,13,14,15 are not nucleotide sequences but amino acids

## Information on patent family members

International Application No  
PCT/US 98/19056

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0666314 A	09-08-1995	CA	2080920 A	21-10-1991
		EP	0537173 A	21-04-1993
		WO	9116457 A	31-10-1991
		US	5527896 A	18-06-1996